Effects of Grazing Soil Fauna on the Functioning and Community Composition of Saprotrophic Basidiomycete Fungi

A thesis submitted to Cardiff University for the degree of Doctor of Philosophy

by

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DECLARATION

This work has not previously been accepted in substance for a degree and is not concurrently submitted in candidature for any degree.

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Summary

Saprotrophic fungi are key regulators of nutrient cycling and re-distribution within woodland ecosystems. They are the primary agents of wood and leaf litter decomposition, and their hyphal networks, which grow throughout the soil-litter interface, represent highly dynamic channels through which nutrients are readily distributed. These networks also represent the primary resource for a huge diversity of mycophagous soil fauna. This study uses soil microcosms to investigate and compares the potential of soil invertebrates, representing the Isopoda, Myriapoda, Acari, Collembola, Tubificida (Enchytraeidae) and Nematoda, to influence mycelial emergence, morphology, extracellular enzyme production and wood decomposition by cord-forming basidiomycetes. While all invertebrates disrupted mycelial growth to some extent, macrofauna (woodlice and millipedes) generally exerted the strongest grazing pressures. By severing thick cords, these larger invertebrates limited mycelial growth and induced the strongest enzymatic responses. In contrast, while the smaller micro- and mesofauna reduced extension rates of some fungal species, their low-intensity grazing also induced compensatory growth responses, stimulating growth of less palatable fungal species. The varying susceptibility of different fungi to grazers also caused grazers to exert selective pressures on fungal communities. By removing entire networks of the most combative fungal species, the woodlouse *Oniscus asellus* prevented the competitive exclusion of three fungal opponents from soil and wood. By stimulating growth of the less competitive fungal species, the nematode *Panagrellus redivivus,* also reversed the outcomes of specific mycelial interactions. Via these two opposing mechanisms soil invertebrates are likely to exert top-down control, influencing the community compositions of saprotrophic fungi. Overall, the effects of grazers on mycelial distribution, decomposition and community compositions were strongly specific, suggesting that the factors which influence invertebrate diversity and community compositions will also indirectly affect mycelial growth and functioning in temperate woodland soils.

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1. General Introduction

1.1. The global soil environment

The Earth's surface is subject to a variety of harsh, highly variable weather conditions. Consequently, the vast majority of terrestrial life, both in terms of species number and biomass, exists below ground (Giller 1996). The activity of this belowground biota, which includes *inter alia* microbes, animals and plant roots, contributes extensively to many of the key physical (regulation of soil structure and edaphic water regimes) and biochemical (nutrient cycling, greenhouse gas production and carbon sequestration) processes which regulate terrestrial ecosystem functioning and support all terrestrial life (Wardle 2002; Wardle *et al.* 2004; Bardgett 2005).

In the absence of light, the major ecosystem energy source below ground is in the form of allochthonous inputs including dead plant material and root exudates. These form an extensive resource and support a variety of highly diverse soil decomposer communities (Cebrian 1999). Approximately 75% of the world's terrestrial carbon (1400–1500 Pg) is stored in this soil organic material (SOM) (Post *et al.* 1982). Decomposition of dead plant material by soil organisms is not only key in the recycling of nutrients for continued primary productivity, but it is also responsible for the annual global release of approximately 50–75 Pg carbon into the atmosphere; several orders of magnitude higher than that of fossil fuel emissions (Schimel *et al*. 1996; Wall *et al.* 2008; Kutsch *et al.* 2009).

1.2 Brief history of soil ecology

Since the earliest civilizations, few things have concerned human populations more than their relations with soil as it provided most of their food and nutrients (McNeill & Winiwarter 2004). 10,000 years ago, Neolithic farmers in Southwest Asia cultivated and enriched soils to produce crops, which supported themselves and their livestock. This relationship between humans and soil is evident in a rich literature on subjects, including soil fertility and management, which dates back to ancient peoples of the Middle East, China, India and the Mediterranean (McNeill & Winiwarter 2004). John

Steinbeck's famous novel, *Grapes of Wrath* (1939) portrays the dependency of humans on soil fertility, as the dust bowl, droughts and changing agricultural practices led to economic turmoil and drove poor families from their homes in the American prairie lands. Despite this historical and literary context, the opacity and complexity of the soil environment have limited our ecological understanding of this belowground habitat and the organisms that maintain it. Traditionally (prior to the last couple of decades), ecologists have portrayed the inhabitants of soil as a black box containing "decomposers" which represent a single trophic level through which all aboveground material is ultimately recycled (see Sudgen *et al.* 2004).

It is only in recent decades that ecologists have begun exploring the complex nature of belowground biological communities. Following advances in a range of analytical techniques including molecular phylogenetics and stable isotopes, interest in soil science is now "booming" (Bardgett 2005) and this has led to significant advances in understanding of the processes influencing, and influenced by, soil organisms. The biological interactions which govern these belowground processes have direct and practical implications for natural and agricultural land management. The increasing interest in soil research is also driven by awareness of climate change and the important role soils play in regulating the carbon cycle, and atmospheric carbon dioxide (CO_2) and methane (CH_4) concentrations (Kutsch *et al.* 2009). A thorough understanding of belowground interactions is important in determining how changes in biological communities, brought about by projected climatic conditions (including elevated temperature and moisture), are likely to influence ecosystem functioning and carbon cycle feedbacks (Wardle *et al.* 2004).

1.3 Forest ecosystem soils

Forests and other wooded biomes cover approximately 39% of the Earth's terrestrial surface area (Whittaker 1975). They occur in tropical, temperate and boreal climate zones, and contain, in the form of living plant material, around 86% of the world's aboveground carbon (Sedjo 1993). As a result of extensive litter inputs, it is estimated that woodland soils contain 73% of the world's soil carbon (Post *et al*. 1982) and can directly influence changes in atmospheric CO_2 concentrations (Schimel 1995; Cao & Woodward 1998). Woody plant material, ranging from individual leaves to entire

woody trees, is distributed heterogeneously across the litter horizon (Rayner & Boddy 1988). In deciduous woodland these nutrient inputs vary throughout the year and commonly form a discontinuous resource. Heavily lignified, this woody litter represents a low quality, recalcitrant resource (Rayner & Boddy 1988). These three features of woody litter (spatially heterogeneous, temporally variable and low nutrient quality) mean that the number of taxonomic groups capable of exploiting it is extremely limited.

Saprotrophic microbes are the primary decomposing agents within temperate and boreal forest ecosystems (Swift & Boddy 1984). Most bacteria and lower fungi rely on labile nutrient sources as they lack the digestive enzymes or penetrative abilities to access heavily lignified resources (Rayner & Boddy 1988). The powerful enzymatic capabilities of basidiomycete and ascomycete fungi to break down complex organic material found in dead wood make them the primary agents of woody litter decomposition (Boddy & Watkinson 1995). These fungi are responsible for most nutrient turnover, decaying fresh resources sufficiently to allow colonization by other decomposer organisms such as lower fungi, bacteria and invertebrates (Lindahl *et al.* 2002). Following resource acquisition, some non-unit-restricted species are capable of producing vast, persistent mycelia that extend throughout the soil in search of fresh litter resources (Boddy 1993). This modular growth enables fungi to overcome the problem of resource heterogeneity but also makes them vulnerable to a vast array of invertebrate species which are capable of exploiting the highly concentrated nutrient sources (Swift & Boddy 1984). Despite the importance of basidiomycete fungi and invertebrates in woody litter decomposition, and the ubiquitous nature of both in temperate woodland soils (Bardgett 2005), significant gaps remain in our understanding of their interactions and implications for fungal-mediated nutrient dynamics.

1.4 Thesis aims

Until now, studies exploring the effects of grazing invertebrates on saprotrophic fungi have been dominated by mycophagous collembola (Boddy & Jones 2008). By ingesting hyphae, these mesofauna can affect the distribution of foraging basidiomycete mycelia (Newell 1984a; Kampichler *et al.* 2004; Harold *et al.* 2005; Tordoff *et al.* 2008). Large

collembola populations can consume entire mycelial systems (Tordoff *et al.* 2006), while lower intensity grazing can induce morphological responses and even stimulate mycelial growth (Hedlund *et al.* 1991; Bretherton *et al.* 2006). Grazing preferences and impacts can vary substantially between collembola species (Tordoff *et al.* 2008), but effects of the vast majority of soil fauna remain unknown. This thesis explores the potential of different soil invertebrate taxa to influence the growth, functioning and composition of saprotrophic basidiomycete fungal communities.

Chapter 2 reviews the literature on interactions between grazing invertebrates and saprotrophic fungi in soil and litter. Specifically, the effects of different grazer species on fungal-mediated nutrient distribution (via propagule dissemination and mycelial growth) and decomposition (via extracellular enzyme production and respiration) are discussed. The potential of grazers to affect saprotrophic fungal community composition is also explored and parallels are drawn with the effects of grazing on mycorrhizal fungi. Knowledge gaps are highlighted; these are then explored in the following experimental chapters. A version of this review has been submitted to *The ISME Journal*.

While the majority of published fungus-invertebrate grazing studies to date have concentrated on the effects of collembola grazing, *Chapter 3* investigates and compares the differential effects of seven common soil invertebrate species on the growth, distribution and wood decomposition by basidiomycete fungi. A version of this study has been published in *Oecologia*, **167,** 535-545.

In aboveground plant-insect interactions it is known that chronology of effect plays a major role in determining the eventual outcome of grazing interactions. In *Chapter 4*, the effects of invertebrate grazing prior to, and following, mycelial emergence from woody resources are compared. This study also highlights the species-specific nature of grazing interactions from an invertebrate and fungal perspective. A version of this study has been published in *Fungal Ecology,* **5,** 333-341.

Previous chapters recorded changes in fungal-mediated decomposition rates as a result of grazing. To understand the physiological mechanisms behind this, *Chapter 5* explores the potential of different grazer populations to influence extracellular enzyme

production by basidiomycete fungi in soil. A version of this study has been published in *Soil Biology & Biochemistry,* **43,** 2060-2068.

Chapter 6 investigates the community consequences of interactions and questions whether the variability in fungal susceptibility to grazing, recorded in previous chapters, is sufficient to allow grazers to exert selective pressures on fungal communities and even alter the outcomes of competitive interactions in soil. This study has been published in *Ecology Letters,* **14,** 1134-1142.

As all these studies have been conducted using pre-determined invertebrate densities, *Chapter 7* describes a short study that was carried out to determine whether the density-dependent effects of soil invertebrates on mycelial development will override the species-specific effects. A version of this study has been published in *Fungal Ecology* (in press, doi:10.1016/j.funeco.2011.07.006).

Finally, *Chapter 8* draws together the salient points from the experimental chapters and, while remembering the limitations of microcosm studies, attempts to extrapolate to what might be expected in the field. Remaining knowledge gaps are highlighted and further avenues of research suggested.

2. Literature review: functional and ecological consequences of saprotrophic fungus-grazer interactions

2.1 Introduction

Woody plant material constitutes between 92 and 99% of above-ground biomass within woodland ecosystems (Swift & Boddy 1984). Of this, approximately 90% escapes herbivory and eventually enters the dead organic matter (DOM) pool, forming the basis of the detritus-based food chain (Cebrian 1999). The extensive lignification and high carbon: nitrogen (C:N) ratio of woody litter makes it a recalcitrant resource (Rayner & Boddy 1988) restricting the organisms capable of exploiting it to saprotrophic fungi and insects (some Isoptera and Coleoptera). A powerful cocktail of lingo-cellulolytic enzymes - capable of the initial deconstruction of complex organic compounds (Sinsabaugh *et al.* 2008) - make saprotrophic basidiomycete and some xylariaceae fungi the primary decomposing agents within woodland ecosystems (Rayner & Boddy 1988; Baldrian & Valášková 2008). Subsequently, saprotrophic Ascomycota and Zygomycota fungi, along with bacterial and animal decomposers, often contribute to the latter stages of decomposition (Cooke & Rayner 1984; Swift & Boddy 1984). During decomposition of litter, the C:N ratio gradually decreases and inorganic nutrients are released into the surrounding environment (Lindahl *et al.* 2002). This 'slow cycling of nutrients' contributes to ecosystem stability, allowing slow-growing plant species to obtain nutrients and ensuring that these nutrients are retained within late succession woodland ecosystems (van der Heijden *et al.* 2008; Fierer *et al.* 2009). Saprotrophic fungi contribute up to 90% of total non-plant respiration (Cooke & Rayner 1984). Consequently, they are considered to be the key regulators of organic matter deconstruction, nutrient cycling and carbon flux between the biosphere and atmosphere (Hättenschwiler *et al.* 2005; Gessner *et al.* 2010).

The progressive degradation of resources after acquisition forces fungi to engage in a continual search for fresh nutrients (Jonsson *et al.* 2005). While unit-restricted fungi (those that are limited to individual pieces of litter) rely on propagule (spores or hyphal fragments) dissemination to encounter new resources, the mycelia of non-unit-restricted species proliferate from, and interconnect, discrete organic resource units. The

filamentous cells of basidiomycetes (mycelia) have an average diameter of 2–10 μm, but can extend up to 80 m within 1 g of dry litter material (Osono 2007) providing structure and support for soils. Hyphal aggregations (known as cords) enable some species (including *Hypholoma fasciculare, Phanerochaete velutina* and *Megacollybia platyphylla*) to extend tens of metres across the soil-litter interface (Thompson & Rayner 1982). Independent translocation of carbon (Cairney 1992), nitrogen (Tlalka *et al.* 2002) and phosphorous (Wells *et al.* 1990) throughout these cords means that woodland soils become highly dynamic environments where nutrients rarely remain concentrated at the point of first introduction (Wells *et al.* 1990; Tlalka *et al.* 2002). Mycelial distribution varies between fungi, depending on morphology and foraging strategy. Short range, exploitative species exhibit a diffuse growth front with little space between foraging hyphae to maximise the likelihood of encountering nearby resources (Boddy 1993). This 'phalanx' morphology (Fig 1A) is the antithesis of the exploitative 'guerrilla' growth (Fig. 1C) where sparsely distributed cords allow fungi to seek-out more substantial woody resources over a larger scale (Boddy 1999). Within these functional groups, foraging system morphology is determined by three related factors: microclimate, resource availability and interspecific interactions (Dowson *et al.* 1988). The latter plays a major role in the release of nutrients from otherwise conservative mycelia (Boddy & Watkinson 1995).

Mycelial extension across the soil-litter interface inevitably leads to encounters with antagonistic microbes. The competitive abilities of basidiomycete and ascomycete fungi generally enable them to overcome and digest competing bacteria and micro-fungi (Boddy 1993) but when two non-unit restricted systems interact they are 'forced' to compete for resources. Inter-, and intra-specific fungal interactions can take place at a distance or following mycelial contact (Boddy 2000). Long-range antagonism occurs through the production of volatile organic compounds, which inhibit the competitor's growth (Wald *et al.* 2004). When hyphal contact cannot be avoided, two combative strategies are employed to kill opposing fungi; mycoparasitism (acquiring nutrients from competitors) and hyphal interference (killing hyphal compartments) (Rayner &

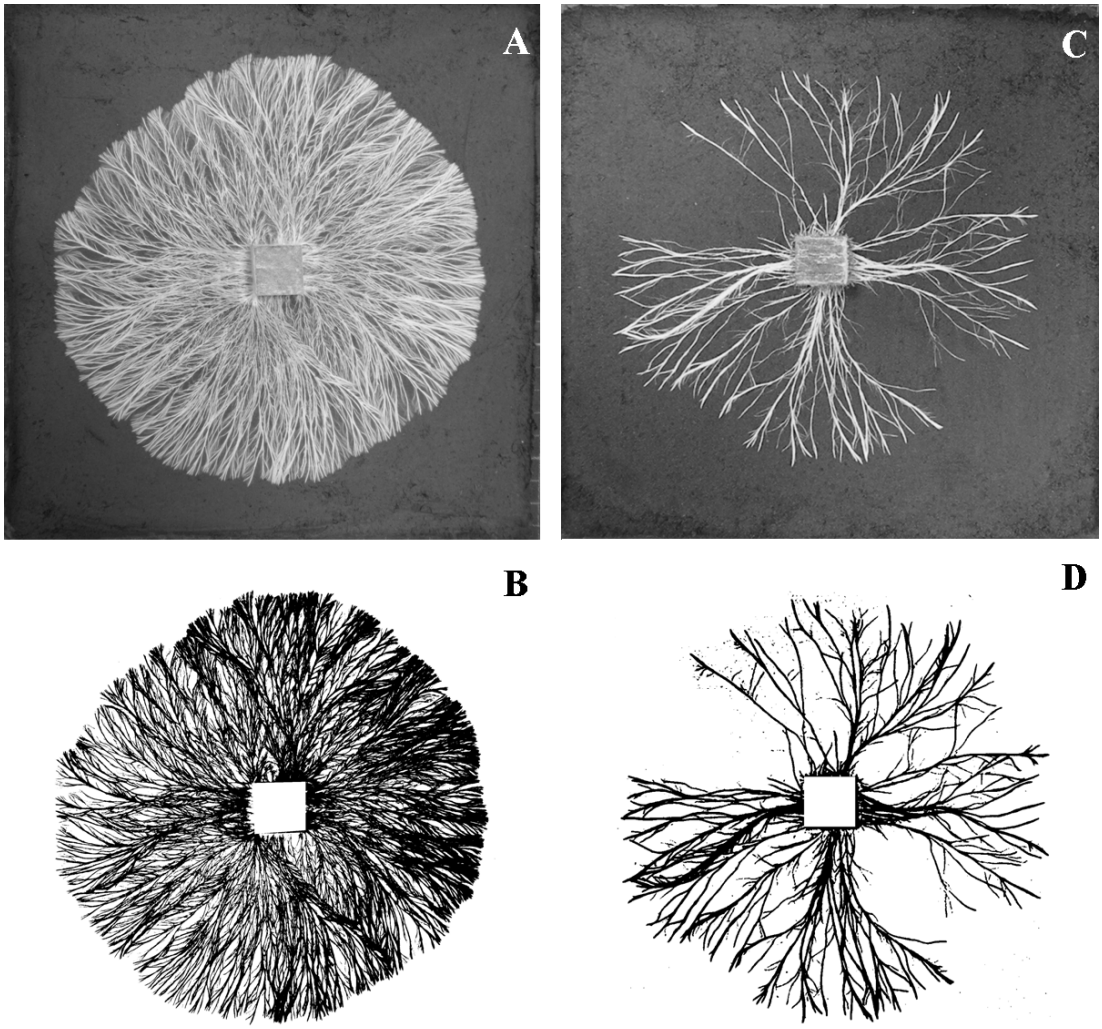

Fig. 2.1: Digital images (A, C) and converted black and white images (B, D) showing growth patterns of fungi exhibiting exploitative, guerrilla (A, B), and explorative, phalanx (C, D) morphologies.

Boddy 1988). When cord-forming basidiomycetes encounter one another, mycelial interactions commonly occur across a wide mycelial front and this 'gross mycelial contact' can be devastating for competing individuals (Boddy 2000). The outcomes of these competitive interactions determine fungal community composition in soil. Species-specific fungal respiration and decomposition rates (Newell 1984b; Hättenschwiler *et al.* 2005; Gessner *et al.* 2010) highlight the importance of these combative fungal interactions in terms of nutrient cycling and soil carbon storage (van der Heijden *et al.* 2008).

The 90 Gt of terrestrial plant biomass entering the soil organic nutrient pool annually (Cebrian 1999) also supports a diversity of fauna unrivalled in any other terrestrial ecosystem (Killham 1994). This has led to soil ecosystems being termed 'the poor man's tropical rainforest' (Usher *et al.* 1979). Soil invertebrates contribute extensively to the functional diversity found within terrestrial ecosystems (Setälä *et al.* 2005), playing various roles in the comminution of litter, which increases the surface area available for microbial colonisation (Bardgett 2005; Wardle *et al.* 2006), and the structuring of microbial community composition (Swift & Boddy 1984). Soil fauna classification is frequently based on size (micro-, meso- or macro-) (Petersen & Luxton 1982) or functional group (Rusek 1998). In terms of nutrient cycling, the most important of these are the arthropods (e.g. collembola, mites, termites, millipedes and woodlice), oligochaetes (earthworms and enchytraeid worms), molluscs (including slugs and snails) and nematodes (Boddy & Jones 2008). The vast majority are primarily mycophagous (Pollierer *et al.* 2009) and possess chitinases for digesting fungal cell walls (Berg *et al.* 2004). Damage to hyphal compartments and production of faecal pellets directly influences the release of labile nutrients into the surrounding soil (Clarholm 1985; Boddy & Watkinson 1995); an important process in the recycling of nutrients to plants (Bardgett & Chan 1999). Invertebrate 'grazing' can also influence basidiomycete growth and physiology (Boddy & Jones 2008). Given the prominent roles of saprotrophic fungi in nutrient decomposition and re-distribution (Boddy 1999; Hättenschwiler *et al.* 2005), grazing represents one of the primary roles of soil fauna on ecosystem functioning and regulation.

The various mechanisms by which invertebrates and saprotrophic fungi interact (trophic and non-trophic) have been reviewed extensively (Lussenhop 1992; Boddy & Jones 2008; Maraun *et al.* 2003). This review focuses on the consequences of grazing interactions for fungal functioning and community ecology (Fig 2.2). Specifically, it explores the effects of grazing soil invertebrates on (i) fungal-mediated nutrient distribution (via mycelia and spores); (ii) mycelial physiology and nutrient cycling (extracellular enzyme production and respiration); and (iii) fungal community composition and diversity.

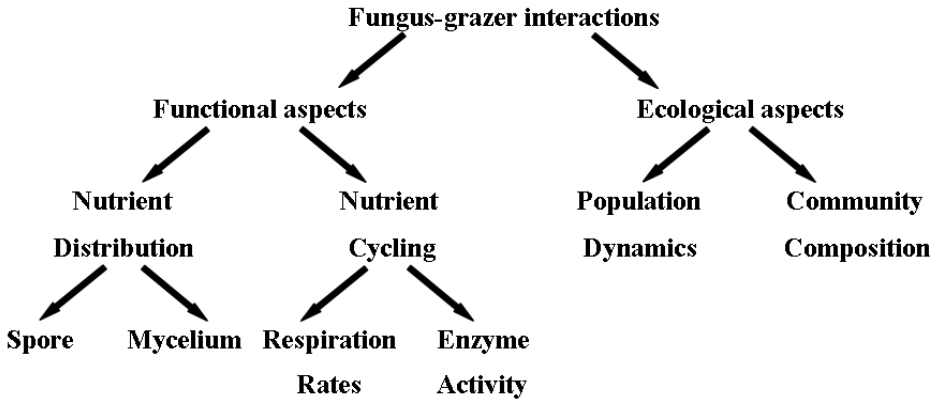

Fig. 2.2: Breakdown of the key functional and ecological aspects of fungus-grazer interactions.

2.2 Mycelial growth and propagule dissemination: redistribution of nutrients

By virtue of their large biomass, and relatively low C:N and C:P ratios, fungi represent the primary nutrient source for soil-dwelling animals (Swift & Boddy 1984; Pollierer *et al.* 2009). Their potential to mineralise organic resources and secrete nutrients also means that fungi regulate the availability of nutrients to plants (Bardgett 2005). As such, fungal spatial organisation effectively reflects the distribution of readily available soil nutrients for plants and animals.

2.2.1 Propagule dissemination

Grazing invertebrates can damage or destroy fungal propagules, but can also act as vectors, assisting in their dispersal. This can be passive - resulting from external adherence of propagules to invertebrate bodies or passage in guts - or active, when fungal propagules are carried in specialised sacs (e.g. mycangia) on, or in, the invertebrate's body. Active dissemination often occurs where a mutualistic symbiotic relationship has evolved such as that between the higher termites (Macrotermitinae) and basidiomycetes in the genus *Termitomyces* (Aanen & Boomsma 2006), attine ants and basidiomycete *Attamyces*, *Leucoagaricus* and *Lepiota* (North *et al.* 1997; Aanen & Boomsma 2006), and *Amylostereum* (Basidiomycota) and woodwasps (Siricidae) (Slippers *et al.* 2003). Such carriage ensures that the mutualistic partners remain together when the invertebrates disperse to new locations. Passive dispersal of fungal propagules by oribatid mites (Renker *et al.* 2005), earthworms (Moody *et al.* 1996), collembola (Visser *et al*. 1987) and enchytraeids (Hedlund & Augustsson 1995) can also result in the vertical and horizontal redistribution of propagules within litter layers.

Passive transport via passage through invertebrate guts is not always successful, as germination and subsequent development can be inhibited. Earthworm (*Lumbricus terrestris* and *Aporrectodea longa*) grazing, for example, reduced or prevented germination of several basidiomycete and ascomycete spores, but propagule survival varied between species; germination of ascomycete, *Chaetomium globosum*, spores was stimulated following ingestion by *A. longa* (Moody *et al.* 1996). This specialised association inferred a selective advantage to the stimulated fungal species following grazing. Similar symbiotic interactions with termites (Mueller & Gerardo 2002) and dipteran larvae (Nuss 1982) have also been found to stimulate spore germination. For some fungi (e.g. basidiomycete species of *Ganoderma*), passage of spores through invertebrate (molluscs in this case) guts is essential to allow successful germination (Nuss 1982).

2.2.2 Mycelial distribution

The extensive mycelial systems of non-unit restricted fungi that grow throughout the rhizosphere represent highly dynamic channels through which nutrients are readily translocated (Boddy 1999; Tlalka *et al.* 2002). The growth and development of these networks are viewed as key processes in the distribution of nutrients within woodland soils (Wells *et al.* 1990; Cairney 2005). Recently, extensive research using collembola and, to a lesser extent, other invertebrates, have highlighted the potential of grazers to influence mycelial distribution (Tordoff *et al.* 2006; Bretherton *et al.* 2006; Wood *et al.* 2006; Tordoff *et al.* 2008). By severing mycelia and ingesting growing hyphal tips, mycophagous collembola (*Folsomia candida*) populations can restrict mycelial extension across soil (Tordoff *et al.* 2006). High-intensity enchytraeid (*Enchytraeus crypticus*) grazing can remove mycelial systems of the nematophagous basidiomycete, *Hirsutella rhossiliensis*, entirely (Jaffee *et al.* 1997). This grazing not only limits the ability of foraging basidiomycetes to encounter new resources but also disrupts the translocation of carbon (Butenschoen *et al.* 2007) and nitrogen (Tordoff *et al.* 2011) throughout mycelial systems. These changes in nutrient partitioning are likely to affect the dynamics and spatial heterogeneity of forest floor nutrients.

Mycelial systems are highly dynamic, and most species show distinct growth responses during grazing. Compensatory growth, similar to that seen in plants during herbivory (McNaughton 1983), has been recorded in a range of fungal species (Hanlon &

Anderson 1979; Hedlund *et al.* 1991; Bengtsson *et al*. 1993; Bretherton *et al.* 2006). This is often characterised by increased mycelial extension rates and branching of hyphae around thick cords (Fig. 2.3). This morphological response is predicted to facilitate increased nutrient uptake to counteract the negative effects of grazing (Bengtsson *et al*. 1993). It could also represent an 'escape response', increasing extension rates into ungrazed regions of soil (Hedlund *et al.* 1991). Fungal responsiveness to grazing varies between functional groups. The exploitative basidiomycetes, *P. velutina* and *H. fasciculare*, were found to increase extension rates during collembola grazing (Bretherton *et al.* 2006), while the explorative forager, *Resinicium bicolor* showed no such response (Tordoff *et al.* 2006). Increased efficiency of soil nutrient uptake by exploitative species (Boddy 2000) may facilitate the increased growth rates as morphology switches from 'guerrilla' to 'phalanx' (Kampichler *et al.* 2004).

Older, more established systems are less responsive to grazing (Tordoff *et al.* 2006). Mycelial contact with new resources often leads to the rapid formation of thick interconnecting cords. These highly sclerotised mycelia are commonly avoided by mesofauna (Kaneko *et al*. 1995) and, as a result, are less susceptible to grazing by collembola (Wood *et al.* 2006). As with many plant species (Molano-Flores 2001), increased accumulation of calcium oxalate crystals – a by-product of lignin decomposition in basidiomycete fungi – by older, more established mycelia is likely to deter grazers further (Shimada *et al*. 1997). Even in the absence of new resources, the interstitial proliferation of hyphae in response to grazing can lead to the formation of cross-links between mycelia, increasing the resilience of remaining networks to further attack (Boddy *et al.* 2010). Early stages of mycelial emergence may, therefore, be a particularly vulnerable and important phase in the development of saprotrophic fungi and the formation of mycelial networks.

Impacts of grazing collembola are density-dependent. Compensatory growth responses are associated with low intensity grazing, while growth is often inhibited at high collembola densities (Bengtsson & Rundgren 1983; Hanlon & Anderson 1979; Bretherton *et al.* 2006). This observation highlights the mechanism of top-down control of fungi by invertebrate predators. Predation of mycophagous collembola (*Folsomia fimetaria*) by predatory mites (*Hypoaspis aculeifer*) limited their grazing potential and,

indirectly, stimulated growth and respiration rates of three ascomycetes in soil compared to fungus only controls (Hedlund & Ohrn 2000). In contrast, factors (abiotic and biotic), which contribute to increased collembola abundance often lead to reduced fungal growth and activity (Lenoir *et al.* 2007).

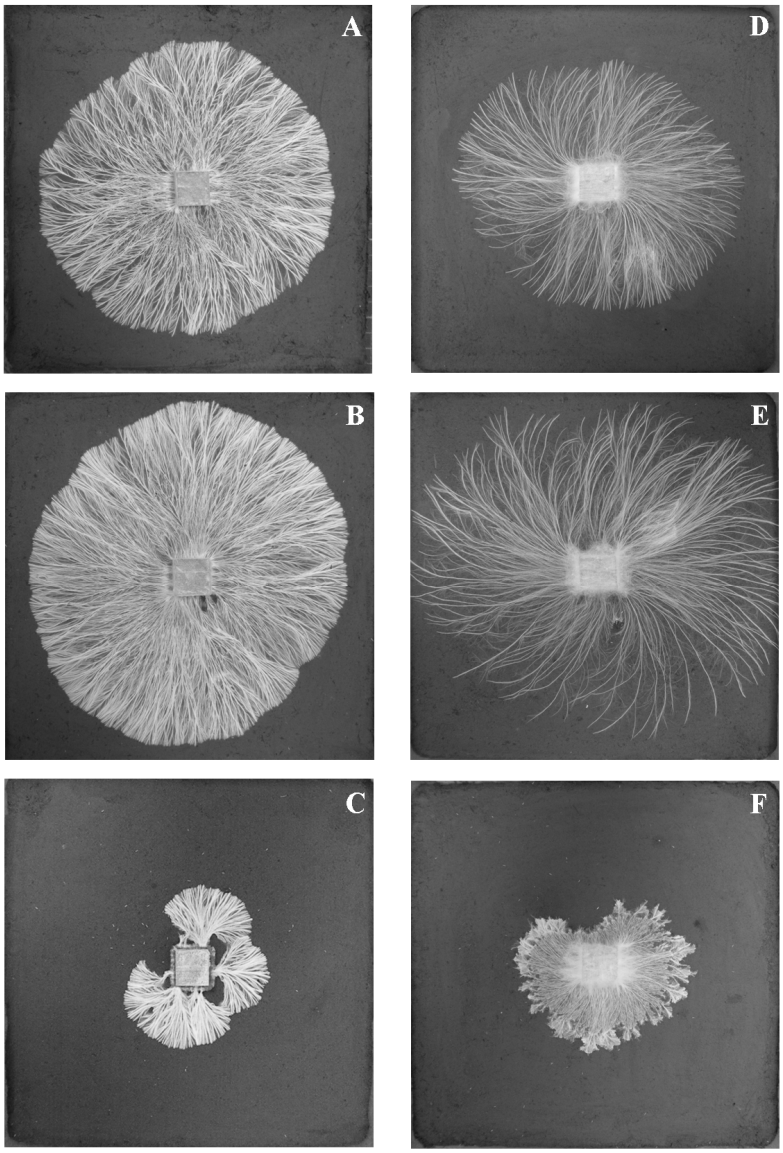

Fig. 2.3: Digital images showing mycelia of *Hypholoma fasciculare* (A, B, C), and *Phanerochaete velutina* (D, E, F) during un-grazed (A and D), low intensity (B and E) and high intensity (C and F) *F. candida* grazing treatments following 10 d of growth from 2 x 2 x 1 cm wood blocks. Low intensity (783 collembola m^{-2}) grazing stimulated early mycelial extension, while high intensity (1,566 collembola m^{-2}) restricted growth of both fungi.

Stark differences in the grazing impacts of different collembola species (Kamplichler *et al*. 2004; Tordoff *et al.* 2008) suggest that collembola community composition may also be an important factor affecting fungal growth and productivity. Although little is known about the effects of different invertebrate species, it is predicted that variation between invertebrate orders is likely to outweigh that within (A'Bear *et al.* 2010). This highlights the potential for changes in collembola community composition, brought

about by global climate change (Jones *et al.* 1998; Wolters *et al.* 2000; Briones *et al.* 2009) to influence fungal-mediated nutrient distribution throughout woodland soils.

2.3 Physiological responses: decomposition and nutrient cycling

By influencing fungal foraging, grazing invertebrates indirectly affect fungal-mediated organic matter decomposition. Their potential to alter fungal physiology (enzyme production and respiration) has more direct consequences for nutrient cycling.

2.3.1 Enzyme production

Hydrolytic enzymes produced by basidiomycete and ascomycete fungi are responsible for the initial step in the deconstruction of plant cell walls and the mineralisation of complex compounds into simple inorganic molecules (sugars, amino acids, NH_4^+, PO_4^{-3}, H_2O and CO_2) which can be assimilated (Baldrian & Valášková 2008; Sinsabaugh *et al.* 2008). Lignocellulytic enzyme production by saprotrophic basidiomycetes colonising leaf litter has been shown to increase during macrofauna (Scheu 1993) and collembola (Parkinson *et al.* 1979) activity. This may be due, in part, to the comminution of litter by soil fauna, but the direct effects of hyphal grazing are likely to be primarily responsible for the enhanced nutrient mineralisation (Osono 2007). Few studies have attempted to separate these indirect (litter comminution) and direct (hyphal grazing) effects. In one such study, collembola (*Onychiurus armatus*) grazing increased specific protease and α-amylase production by the zygomycete, *Mortierella isabellina*, growing in agar (Hedlund *et al.* 1991). The nematode, *Panagrellus redivivus*, also increased protease production by the basidiomycete, *P. velutina*, although the opposite effect was recorded under *Stereum hirsutum* networks (Dyer *et al.* 1992). Contrasting enzymatic responses of different fungal species suggest that the impacts of soil fauna on fungal-mediated nutrient mineralisation are not uniform, but determined by fungal community composition.

2.3.2 Decomposition and respiration

The enzymatic responses of saprotrophic fungi during grazing will facilitate nutrient uptake with direct consequences for wood and leaf litter decomposition. Generally, invertebrate activity stimulates litter decomposition (Swift & Boddy 1984, Wardle 2006; Wall *et al*. 2008) although, once again, most studies have not separated the direct and indirect effects on fungal activity. Recent microcosm studies indicate that, when

restricted to feeding on extra-resource mycelia growing in soil, collembola (*F. candida*) can reduce decay rates of basidiomycete-colonised beech (*Fagus sylvatica*) wood (Tordoff *et al.* 2006; 2008). By reducing fungal biomass and activity, high intensity grazing can limit the ability of fungi to digest wood (Boddy & Jones 2008). At lower collembola densities, the stimulation of fungal growth and enzyme production (Hedlund *et al.* 1991) are, however, likely to have the opposite effect on wood decay.

During decomposition, fungal respiration is responsible for the release of CO_2 from litter resources. Through this process, fungi contribute extensively to total respiration in woodland soils (Post *et al.* 1982; Bardgett 2005). By modifying fungal productivity (growth, enzyme production and nutrient uptake) grazers influence the flux of carbon between the terrestrial and atmospheric carbon pools. Mycelial respiration is likely to be directly linked to biomass and enzyme activity (Table 2.1). Effects of grazers on fungal respiration are, therefore, density-dependent (Hanlon & Anderson 1979). At high densities, collembola reduce fungal respiration (Bardgett *et al.* 1993), while low intensity grazing stimulates CO_2 production (Hanlon & Anderson 1979; Visser *et al.* 1981; Kaneko *et al.* 1998). Similar density-dependent trends have been recorded during enchytraeid (Hedlund & Augustsson 1995) and oribatid mite (Kaneko *et al.* 1998) grazing. The potential of grazers to influence soil decomposition, nutrient mineralisation and carbon storage is, therefore, likely to depend on grazing intensity. While this appears to be directly related to invertebrate density (Bretherton *et al.* 2006), the importance of species identity is, as yet, unknown (Tordoff *et al.* 2008).

2.4 Fungal community structure

2.4.1 Direct trophic effects

The majority of soil fauna are generalists, capable of exploiting a variety of microbial resources within highly diverse and heterogeneous environments (Setälä *et al.* 2005). Such indiscriminate feeding can have major consequences for fungal species richness. Wicklow and Yacom (1981; 1982), for example, found that the number of coprophilous fungal species on rabbit faeces was dramatically reduced during grazing by sciarid fly, *Lycoriella mali*, larvae. By ingesting entire micro-fungi, indiscriminate grazers reduced the amount of competition between fungi; remaining fungi were released from the competitive stress which led to increased rates of organic material decomposition. By

reducing competition between fungi, indiscriminate grazers may stimulate the cycling of nutrients and efflux of CO_2 from soil. This proposed mechanism for stimulated decomposition has, however, been contradicted by more recent studies which report facilitative interactions between microbial species leading to a positive relationship between the number of fungal species and decomposition rate (Tiunov & Scheu 2005a; Hättenschwiler *et al.* 2005). It is, therefore, likely that the effects of indiscriminate grazers will vary between habitats, depending on the level of functional redundancy within the local microbial community.

Despite their polyphagous nature most mycophagous fauna do display distinct feeding preferences for nutritious or palatable fungi (Newell 1984b; Klironomos *et al.* 1992; Maraun *et al.* 2003). In aboveground systems, selective feeding by herbivores and predators can influence plant and animal community composition. Preferential soil invertebrate grazing is likely to exert comparable selective pressures on belowground fungal communities, favouring the growth of less palatable species. This process can be important for fungal communities during early stages of decomposer succession (soon after litter resources have become available and been colonised by microbes) as late-succession fungi are often less susceptible to grazers (Lussenhop 1992). Preferential grazing by the collembola, *F. candida*, on primary saprophytes led to faster replacement by secondary saprophytes on spruce and fir needles (Klironomos *et al.* 1992). By restricting the less competitive species, collembola facilitated fungal succession in decaying litter. Grazers also regulate this process via an opposing mechanism – stimulation of the dominant competitor. In soil microcosms containing two competing basidiomycetes, grazing by *F. candida* stimulated growth of the dominant species, *P. velutina*, over its opponent, *H. fasciculare* (Rotheray *et al.* 2011). This supported previous studies where collembola reinforced the outcomes of competitive mycelial interactions but did not alter the eventual outcomes of competitive interactions (Parkinson *et al.* 1979; Whittaker 1981; McLean *et al.* 1996).

Table 2.1: Effects of invertebrate grazers on biomass, respiration and enzyme activities of individual saprotrophic fungi growing in microcosms. Fungal phyla include Basidiomycota (B), Ascomycota (A) and Zygomycota (Z).

Fungal taxon	Fungal species	Grazer taxon	Effects			Reference
			Biomass	**Respiration**	**Enzyme production or decomposition**	
B	*Coriolus versicolor*	Collembola	increased at low density negative at high density	increased at low density decreased at high density		Hanlon & Anderson 1979
B	various	Collembola	decreased			Leonard & Anderson 1991
B	*Phanerochaete velutina*	Nematoda	increased		increased	Dyer *et al.* 1992
B	*Hypholoma fasciculare*	Collembola	increased at low intensity decreased at high intensity			Kampichler *et al.* 2004
B	*Hypholoma fasciculare*	Collembola	decreased			Harold *et al.* 2005
B	various	Collembola	decreased		decreased	Tordoff *et al.* 2006
B	*Phanerochaete velutina*	Collembola	decreased			Wood *et al.* 2006
B	*Phanerochaete velutina*	Collembola	decreased at high density increased at high density increased following grazing	decreased at high density increased at high density		Bretherton *et al.* 2006
B	various	Oligochaete	decreased			Butenschoen *et al.* 2007
B	various	Collembola	decreased		decreased	Tordoff *et al.* 2008
B	*Phanerochaete velutina*	Collembola	decreased			Boddy *et al.* 2010
B	various	Collembola Oribatida	decreased neutral			A'Bear *et al.* 2010
A/B	*various*	Collembola	increased at low density decreased at high density	increased		Ineson *et al.* 1982
A/B	various	Collembola	increased at low density decreased at high density	increased at low density decreased at high density		Kaneko *et al.* 1998
		Oribatida	increased at low density decreased at high density	increased at low density decreased at high density		

A	*Botrytis cinerea*	Collembola	increased at low density decreased at high density	increased at low density decreased at high density		Hanlon 1981
A	*Phoma exigua*	Collembola	decreased	decreased		Bardgett *et al.* 1993
A	various	Collembola	positive	increased		Bengtsson *et al.* 1993
A	*Hirsutella rhossiliensis*	Enchytraeid	decreased			Jaffee *et al.* 1997
A	various	Collembola	increased at low density decreased at high density	increased at low density decreased at high density		Hedlund & Ohrn 2000
Z	*Mortierella isabellina*	Collembola	decreased increased following grazing	decreased increased following grazing		Bengtsson & Rundgren 1983
Z	*Mortierella isabellina*	Collembola	increased		increased	Hedlund *et al.* 1991
Z	*Mortierella isabellina*	Enchytraeid	increased at low density decreased at high density	increased at low density decreased at high density		Hedlund & Augustsson 1995

If removing the less competitive fungus can stimulate rates of species turnover, selective grazing of the dominant competitor is likely to have the opposite effect, reversing the outcomes of fungal interactions and driving changes in fungal species composition and diversity. Newell (1984a) provided some evidence for this, showing that collembola (*Protaphorura aurantiaca*) grazing differentially affected the relative abundances of two basidiomycetes in Sitka spruce (*Picea sitchensis*) litter. Selective grazing of *Marasmius androsaceus* increased the number of needles colonised by the less competitive fungus, *Mycena galopus* (Newell 1984b). This did not lead to the complete replacement of the dominant competitor, but suggested that grazers may exert selective pressures and influence fungal community structure.

Despite numerous studies reporting preferential invertebrate grazing for soil fungi (see Maraun *et al.* 2003 and references therein), none have confirmed that grazing on the dominant fungal competitor can reverse the outcomes of combative interactions. One proposed explanation is that, despite their capacity to influence fungal growth and physiology, the impacts of soil fauna may not be extensive enough to alter fungal competitive abilities (Wardle & Yeates 1993). By virtue of their large biomass, biochemical and structural defences, fungi may be able to withstand localised grazing events, redistributing and conserving nutrients throughout vast mycelial networks (Boddy 1999). The limited representation of micro- and macrofauna in empirical studies may also explain the reported lack of grazer control. Larger invertebrates have been shown to exert stronger pressures on fungal activity (Bradford *et al.* 2002) while the abundance of mycophagous microfauna in woodland soil (Petersen & Luxton 1982) highlights their potential to influence fungal communities (Lussenhop 1992). Investigating the effects of a wider range of soil fauna may be instrumental in identifying the potential of grazers to modify fungal community composition and related ecosystem functioning.

2.4.2 Non-trophic effects

Perhaps the most extreme example of invertebrates influencing fungal community composition is found with the mutualistic symbiotic relationships of higher termites (*Macrotermitinae* spp.) and basidiomycetes (*Termitomyces* genus), and ants and basidiomycetes (*Attamyces*, *Leucoagaricus* and *Lepiota* spp). In both systems, the invertebrates maintain monocultures of the fungi by antibiotic secretions and physical

grooming (Currie *et al.* 1999; Mueller & Gerardo 2002; Aanen & Boomsma 2006). Invertebrates also influence fungal community composition through differential survival of ingested spores; the stimulated germination of *C. globosum* spores following ingestion by *A. longa*, for example, conferred a selective advantage over competing ascomycete fungi damaged or digested by grazing (Moody *et al.* 1996). While grazing will not influence the competitive abilities of germinating fungi, the short-term selective advantage and rapid establishment of stimulated species is a key process influencing fungal species composition and activity.

2.5 Comparisons with mycorrhizal fungi

There are at least six categories of association between mycorrhizal fungi and plants in which fungi usually facilitate the uptake of nitrogen and phosphorus by plants in exchange for organic carbon (Smith & Read 2008). Soil faunal grazing can influence the development of plant-mycorrhizal associations, as well as the transfer of nutrients between symbionts (Fitter & Sanders 1992; Gange 2000; Gange & Brown 2002). A number of parallels can be drawn between the influence of grazers on saprotrophic and mycorrhizal systems: (i) spore ingestion can have positive and negative effects on spore distribution and subsequent germination, depending on the grazer-fungus combination (Harinikumar & Bagyraj 1994); (ii) severing of hyphae can restrict growth and nutrient translocation throughout hyphal networks (Klironomos & Kendrick 1995; Tuffen *et al.* 2002); and (iii) grazing can have positive or negative effects on fungal activity (Klironomos & Ursic 1998; Gormsen *et al.* 2004) with direct consequences for nutrient mineralisation and primary productivity. As with saprotrophic fungi, grazing effects are density-dependent. High intensity grazing can reduce mycorrhizal activity but low intensity grazing can stimulate growth, directly increasing plant productivity (Klironomos & Ursic 1998). Contrasting effects of earthworm (*Lumbricus rubellus*) and collembola (*F. candida*) populations suggest that effects are also taxon-specific (Gormsen *et al.* 2004).

Invertebrate preference for saprotrophic over mycorrhizal fungi represents a key difference between the effects of grazers on these two major fungal groups. When provided with a choice, invertebrates consistently prefer, and reproduce more successfully on, saprotrophic than mycorrhizal fungi (Klironomos *et al.* 1999; Gange 2000). As a result, in complex, multi-species environments soil fauna commonly stimulate mycorrhizal growth by suppression of

competing or inhibitory fungi (Klironomos & Kendrick 1995). Plant pathogenic fungi are also generally preferred to mycorrhizal species, highlighting further the positive effects of grazers on plant productivity (Lussenhop 1992). Variation in the susceptibility of major fungal groups can lead to shifts in fungal community composition. Collembola grazing, for example, destabilised the saprotrophic fungal community, making it more susceptible to the negative effects of the competing arbuscular mycorrhizal species, *Glomus mosseae* (Tiunov & Scheu 2005b). Changes in invertebrate abundance, composition and activity are likely to have greater implications for saprotrophic than mycorrhizal fungal communities.

2.6 Conclusions and future directions

In recent years there has been extensive debate concerning how changes in climate, land use and pollution are likely to influence woodland ecosystem functioning and soil carbon storage (e.g. Davidson & Janssens 2006; Bradford *et al.* 2008; Allison *et al.* 2010; Bradford *et al.* 2010). Environmental changes are likely to have a number of direct and indirect effects, mediated through changes in the detritus-based biological community, which alter the cycling and retention of nutrients. The density-dependent impacts of soil fauna on saprotrophic fungi suggest that factors that contribute to an increase in soil invertebrate abundance are likely to reduce fungal-mediated nutrient translocation, decomposition and CO_2 efflux into the atmosphere. In contrast, a reduced abundance of mycophagous invertebrates is likely to stimulate mycelial growth and respiration. It is currently unclear whether global climate change will have a positive or negative effect on soil faunal abundance. Increased precipitation and temperature are predicted to reduce moisture limitation in many woodland ecosystems (including temperate and boreal) but effects are likely to vary drastically between biomes (Briones *et al.* 2009; Day *et al.* 2009). Such changes may also influence the trophic, and competitive interactions between invertebrate species, and so the overall effects on grazer abundance remain unclear.

Species-specific environmental tolerances of soil fauna suggest that changes in taxonomic composition of soil biotic communities are more predictable (Jones *et al.* 1998; Wolters *et al.* 2000). The differential effects of collembola on mycelial development (Tordoff *et al.* 2008; Kampichler *et al.* 2004) suggest that predicted changes in collembola species composition will cascade to lower trophic levels, driving changes in fungal dominance and activity (Jones *et al.* 1998). Variation between invertebrate functional groups is, however, likely to be greater

than that within groups (Bradford *et al.* 2002). Only two studies have, to date, compared the effects of different invertebrate taxa on saprotrophic activity, revealing slight variation in the effects of oribatid mites and collembola on mycelial growth and respiration (Kaneko *et al.* 1998; A'Bear *et al.* 2010). Investigating and comparing the effects of a wider range of soil invertebrate taxa, including micro-, meso- and macrofauna, on saprotrophic fungi is a vital step necessary to enhance understanding of the the biotic factors affecting mycelial growth and functioning. Such studies could also allow the identification of the most influential and, therefore, functionally important, invertebrate species and shed light on the apparent functional redundancy in the belowground decomposer system.

Although numerous studies have highlighted the potential of grazers to influence fungal species compositions (Newell 1984b; Lussenhop 1992), no empirical study has shown that selective grazing can reverse the outcomes of competitive fungal interactions in soil or litter. The lack of recorded top-down determination of fungal community composition may relate to the limited numbers of invertebrate taxa studies. It is possible that the greater grazing intensity associated with macrofauna (Bradford *et al.* 2002) will exert stronger pressures on fungal communities than the smaller micro- and mesofauna. Species-specific growth, decomposition and respiration rates of saprotrophic fungi highlight the importance of understanding the potential of invertebrates to influence fungal community composition. Investigation and comparison of interactions between fungi and grazers, across a range of species, with increasing levels of community complexity, is essential to understand the importance of grazing interactions for saprotrophic fungal functioning and ecology.

This thesis aims to investigate and compare the effects of invertebrate species, representing some the most common taxa in temperate UK woodlands, on: (i) the growth and morphology of saprotrophic cord-forming basidiomycete fungi in soil; (ii) emergence and establishment of mycelial cords growing from wood resources; (iii) extracellular enzyme production by individual mycelial systems in soil; and (iv) the outcomes of competitive inter- and intraspecific mycelial interactions. Such an understanding may provide valuable insights into the mechanisms governing various belowground processes and the potential for predicted changes in biotic communities, brought about by climate and land use changes, to influence fungal-mediated nutrient cycling and distribution in woodland soil.

3. Species-specific effects of soil fauna on fungal foraging and decomposition

3.1 Abstract

Saprotrophic basidiomycete fungi are primary decomposing agents in terrestrial soils. Their mycelial networks play an important role in nutrient mineralisation and distribution, but are also nutritious resources for various soil invertebrates. Global climate change is predicted to alter the diversity and community composition of these soil fauna. To understand whether changes in invertebrate species diversity are likely to affect fungal-mediated decomposition, this study compared the grazing potentials of different invertebrate taxa and functional groups. Specifically, the grazing impacts of seven invertebrate taxa on the growth, spatial distribution and wood decay rates of six basidiomycete fungi growing from beech wood blocks in soil microcosms were explored. The consequences of grazing were both taxon- and species-specific. Generally, macrofauna caused the greatest damage, while meso- and microfauna often stimulated mycelial growth. Invertebrate size, preferences and population dynamics are likely to influence grazing potentials. Effects of grazing varied between fungi, with mycelial morphology and biochemistry possibly influencing susceptibility. Heavy grazing indirectly increased fungal-mediated wood decomposition. Changes in invertebrate community composition are predicted to have consequences for fungal growth, activity and community structure in woodland soils. Abiotic climate change factors including CO_2 and temperature affect mycelial productivity directly but the indirect effects, mediated through changes in the soil invertebrate community, may be equally important in controlling ecosystem functioning.

3.2 Introduction

Cord-forming basidiomycete fungi are among the primary decomposing agents in temperate and boreal forest ecosystems. They are one of few groups of organisms which possess the ligno-cellulytic enzymes capable of degrading herbaceous and woody litter cell walls (Baldrian & Valášková 2008). Mycelial aggregations, known as cords, extend from and interconnect discrete woody resources enabling nutrients to be obtained and reallocated throughout vast fungal networks (Boddy & Watkinson 1995). Translocation of various nutrients along these cords results in highly dynamic soil environments where nutrients rarely remain concentrated at the point of first introduction (Gessner *et al.* 2010). The distribution of cords throughout the soil-litter interface is variable, and determined by three related factors: microclimate, resource availability and interspecific interactions with antagonistic soil organisms (Dowson *et al.* 1988). The latter play a vital role in the release of nutrients from otherwise conservative mycelia (Boddy & Watkinson 1995). Given the vital role of fungi in terrestrial ecosystem structure and functioning (Hättenschwiler *et al.* 2005; Gange *et al.* 2007), and their influences in human-related activities (Deacon 2006), understanding the consequences of decomposer interactions on fungal growth and functioning is essential.

Soil invertebrates contribute extensively to both functional and species diversity within terrestrial ecosystems (Setälä *et al.* 2005), and are vital to efficient ecosystem function and regulation (Bardgett & Chan 1999; Wardle *et al.* 2004; Hättenschwiler *et al.* 2005). Numerically, and in terms of ecosystem processes, the most important soil fauna include Nematoda, Oligochaeta (earthworms and enchytraeid worms) and Arthropoda (woodlice, collembola, oribatid mites and millipedes) (Bardgett 2005). Several of these 'decomposer invertebrates' are primarily mycophagous and are known to possess chitinases for digesting fungal cell walls (Berg *et al.* 2004). Numerous studies (Bardgett & Chan 1999; Bradford *et al.* 2002; Mitschunas *et al.* 2006; Staddon *et al.* 2010) have highlighted the potential of mycophagous invertebrate 'grazers' to influence fungal-mediated nutrient cycling, decomposition and carbon storage, but the complexity of soil ecosystems has limited our understanding of individual species and interaction effects. Exploring the outcomes of specific soil interactions is vital to improving our understanding of below-ground processes, and their implications for above-ground functioning and productivity (van der Putten & van der Putten 2010).

Empirical studies of soil fungus-invertebrate interactions have generally centred on collembola activities. By ingesting hyphae, mycophagous collembola affect the distribution and activities of foraging basidiomycetes (Bretherton *et al.* 2006; Boddy & Jones 2008; Tordoff *et al.* 2008). Expanding populations of these highly fecund mesofauna can consume entire mycelial systems (Tordoff *et al.* 2006), while lower intensity grazing can induce morphological responses and even stimulate mycelial growth (Hedlund *et al.* 1991). Selective grazing by collembola can alter the outcome of combative fungal interactions, influencing species dominance and decomposition rates within microbial communities (Newell 1984a; Klironomos *et al.* 1992). Grazing preferences and impacts can vary substantially between collembola species (Tordoff *et al.* 2008).

Collembola are, however, only one of numerous soil invertebrate taxa with the potential to influence the distribution and activity of fungal mycelia. Grazing nematodes, earthworms, enchytraeids and mites affect fungal biomass and respiration (Seastedt 1984; Maraun *et al.* 2003), but their effects on basidiomycete spatial distribution, foraging and decomposition rates remain unknown. Only a limited number of studies have investigated the impact of macro-detritivores such as millipedes (Myriapoda) and woodlice (Isopoda) on fungal activity. In one of the few studies that have, Bradford *et al.* (2002) manipulated assemblages of micro-, meso- and macrofauna to compare impacts on microbial decomposition and primary productivity. Decomposition rates increased in macrofauna communities, but the concurrent increase in grazing of mycorrhizal fungi prevented any increases in primary productivity. The importance of investigating and comparing the impacts of micro-, meso- and macrofauna species on fungal growth and activity has been identified as essential to understanding the role of species or functional groups on nutrient cycling and microbial community structure (Butterfield 1999; Mitschunas *et al.* 2006).

In the present study, the grazing impacts of seven soil invertebrate species on mycelial distribution of six basidiomycetes (three strains (genetically distinct individuals) of *Hypholoma fasciculare* (DD2, DD3 and JH) (Huds.: Fr.), and one strain each of *Resinicium bicolor* (Abertini and Schwein.: Fr.), *Phanerochaete velutina* (DC.: Pers.) and *Phallus impudicus* (L.: Pers)) were investigated. Consequences of extra-resource mycelial grazing for wood (*Fagus sylvatica*) decomposition rates were also explored. Representative species of the Isopoda, Myriapoda, Acari, Collembola, Tubificida (Enchytraeidae) and Nematoda were used, based on the abundance of these taxa in soil collected from Coed Beddick Enclosure,

Tintern, UK (NGR 352800, 201800, 51° 41′ 48.37″ N, 2° 40′ 53.11″ W). The invertebrate pool represented micro-, meso- and macrofauna species, with a range of different feeding mechanisms; differentially sized chewing mandibles and maxillae were represented in most meso- and macrofauna, while the nematodes utilised penetrating stylets to access hyphal contents. Different strains of *H. fasciculare* allowed investigation of both inter- and intraspecific differences in mycelial development. Temporal changes to mycelial extension, hyphal coverage and fractal dimension were compared across invertebrate treatments. It was predicted that: (i) invertebrate grazers would have taxon-specific impacts on growth and morphology of individual fungi; (ii) soil macrofauna, along with meso- and microfauna, would feed on, and affect the spatial distribution of foraging basidiomycetes; (iii) mycelial responses to grazing would be species-specific, but not strain-specific, with respect to fungi; and (iv) invertebrate grazers would differentially affect fungal-mediated wood decay rates.

3.3 Materials and methods

3.3.1 Invertebrate culturing

Millipedes, *Blaniulus guttulatus* (Fabricius 1798) (Myriapoda, Julida, Blaniulidae), and woodlice, *Oniscus asellus* Linnaeus 1758 (Isopoda, Oniscidae) and *Porcellio scaber* Latreille 1804 (Isopoda, Porcellionidae), collected from Coopers Field, Bute Park, Cardiff (NGR 317819 176785, 51° 29′ 20.4″ N, 3° 11′ 20.4″ W), were cultured in plastic containers with moistened filter paper and decaying wood blocks. Collembola, *Folsomia candida* Willem 1902 (Collembola, Isotomidae, Cardiff University Collembola Culture), and oribatid mites, *Euzetes globulus* (Nicolet 1855) (Acari, Oribatida, Euzetidae), extracted using Tüllgren funnels from soil collected to a depth of 10 cm from deciduous woodland in the Coed Beddick Enclosure, Tintern, were cultured in 0.6 l culture pots on a medium of 90% plaster of Paris (Minerva Dental, Cardiff, UK) and 10% charcoal (Sigma, Poole, UK). Pots had vented lids. All cultures were kept in the dark at room temperature. The substrate was kept moist using de-ionised water (DH_2O), and the collembola were fed weekly with dried baker's yeast (Spice of Life, Cardiff, UK).

Nematodes, *Panagrellus redivivus* (Linnaeus 1767) (Rhabditida, Panagrolaimidae; supplied by the UK Parasitology Group, Aberystwyth University, UK) were maintained on 35 g autoclaved porridge oats moistened with 60 ml distilled water (121°C for 20 min) in 500 ml jars. Enchytraeids, *Enchytraeus crypticus* Westheide and Graefe 1992 (Tubificida,

Enchytraeidae; supplied by the National Environmental Research Institute of Denmark, Department of Terrestrial Ecology) were cultured on agar medium containing 13.6 g Bacti-Agar No. 1, 772 ml DH_2O, 6 ml 0.1 M $NaHCO_3$, 6.4 ml 0.01M KCl, 8 ml $CaCl{\cdot}2H_2O$ and 8 ml concentrated $MgSO_4$. 'Clean' nematode and enchytraeid suspensions were obtained using wet funnel extraction (Southwood & Henderson 2000). Following extraction, worms were washed for 60 mins in a solution of 5 ppm benomyl and 30 ppm chlorotetracycline, to reduce fungal and bacterial contamination, respectively. Cleaned worms were rinsed in 100 ml deionised water (DH_2O) prior to use.

3.3.2 Fungal culturing and inoculum preparation

All fungal strains (Cardiff University Collection) were sub-cultured on 2% malt extract agar (MEA; 20 g^{-1} Munton and Fiston malt, 15 g^{-1} Lab M agar no. 2) in non-vented 9 cm diameter Petri dishes. Freshly-felled beech (*F. sylvatica*) wood was cut into blocks (2 x 2 x 1 cm) and stored at -18°C. Prior to use, wood blocks were soaked in DH_2O before being autoclaved at 121°C for 20 minutes in double, sealed autoclave bags. The process was repeated twice over 24 h. Sterilized wood blocks were placed onto prepared fungal cultures, sealed with Nescofilm®, and incubated in a dark constant temperature (CT) room at 21°C for three months prior to use.

3.3.3 Microcosm preparation and inoculation

Soil was collected as above from the Coed Beddick Enclosure. After sieving on site (10 mm mesh), the soil was air-dried, sieved through a 2 mm mesh and frozen at -18 °C for 24 h. Soil was rehydrated with 340 ml DH_2O and transferred (200 g; -0.012 MPa) to square, 24 x 24 cm bioassay dishes, smoothed and compacted to about 5 mm depth. 275 soil trays allowed five replicates for each of the 42 interactions (6 fungi x 7 grazers), the six fungus-only, and seven invertebrate-only controls. Wood blocks were cleaned of surface mycelia and excess agar using a spatula, before being centrally placed onto soil trays. Five extra wood blocks from each fungal strain were measured before being dried in the oven at 85°C for 48 h. These blocks were then re-weighed to estimate mean wood density (dry weight/fresh volume; g cm^{-3}) at Day 0.

After inoculation, the weight of each tray and inoculum was recorded, and used as a guide when re-wetting; DH_2O was sprayed evenly across the soil until each tray reached its original mass. Trays were stacked and sealed in polythene bags to reduce water loss, and incubated at

21˚C and 70% humidity. When mycelia in 50% of the trays for each fungal strain had reached 8 cm diameter (mean of two measurements: largest and smallest areas of growth), any foreign micro-fungi were removed with forceps and invertebrates introduced around the mycelium on un-colonised regions of soil.

3.3.4 Invertebrate numbers and size selection

Soil invertebrate numbers used in microcosms were based on densities recorded from Tüllgren funnel extractions (see above). Although at the lower end of the range of reported mean field densities (Peterson & Luxton 1982; Topp *et al.* 2006) experimental numbers (Table 3.1) were considered appropriate to the restrictive 2-dimensional (2-D) microcosm environment used in the current study. Invertebrates, other than nematodes, were size-selected (by passing them through a series of metal sieves of a known pore size we could select the appropriate size range) following Tordoff *et al.* (2006) and Bradford *et al.* (2002).

3.3.5 Microcosm study progression and harvesting

Trays were randomly repositioned weekly within the CT rooms over the 80 d period. These were photographed before invertebrate addition, and then after 2, 4, 8, 16, 24, 38, 52, 66 and 80 d using a Nikon Coolpix 57000 camera, mounted on a stand at a height of 40.5 cm. On completion, macro-invertebrates were removed and counted by hand. The contents of trays containing mesofauna (including enchytraeids) were placed into a Tüllgren funnel and collected invertebrates were counted under 20x magnification. Nematodes were extracted by

Table 3.1: Selected size range (except *P. redivivus)*, field density range, experimental numbers and densities of the seven invertebrate species added to soil microcosms.

	Size range	Field density range (m^{-2})	Experimental numbers	Experiment density (m^{-2})
Oniscus asellus	≥ 0.5 cm	35-630 (Topp *et al.* 2006)	5	83
Porcellio scaber	≥ 0.5 cm	35-630 (Topp *et al*. 2006)	5	83
Blaniulus guttulatus	≥ 0.5 cm	15-230 (Petersen & Luxton 1982)	5	83
Folsomia candida	200-400 µm	100-67 x 10^4 (Petersen & Luxton 1982)	60	783
Euzetes globulus	200-800 µm	300-2 x 10^5 (Petersen & Luxton 1982)	60	783
Enchytraeus crypticus	100-400 µm	0-82 x 10^4 (Petersen & Luxton 1982)	60	783
Panagrellus redivivus	NA	8 x 10^3 - 3 x 10^7 (Petersen & Luxton 1982)	1000	16.6 x 10^3

spraying the soil surface with DH_2O and runoff containing nematodes was collected in a 200 ml beaker following Dyer *et al.* (1992). Nematode populations were estimated using a Sedgewick-Rafter counter. Final wood block density was determined and subtracted from initial wood densities for each fungal strain to estimate woody decay rate (g cm^{-3} d^{-1}).

3.3.6 Image capture analysis

Images were analysed using IMAGEJ (National Institute of Health, USA). A 2 cm calibration line was drawn electronically using a ruler next to each tray. The edge of the soil tray and wood block were removed by windowing and the resulting image converted to black and white (8-bit), and then to binary with a manually set threshold. The mycelia and soil were indicated by red and black pixels, respectively, allowing hyphal coverage (cm^2) to be determined (proportion of red pixels). Mycelial extension was calculated by measuring the mean length (cm) of eight straight lines drawn (at 45° angles to each other) from the centre of the wood block to hyphal tips. Extension rate (cm d^{-1}) was recorded for each fungal strain until mycelia in any replicate reached tray edges. Mass fractal dimensions (D_{BM}) were estimated using the box count method. This provided a quantitative value describing mycelial space-filling and branching (Boddy & Donnelly 2008).

3.3.7 Statistical analysis

Radial extension was compared across treatments by Analysis of Covariance (ANCOVA; General Linear Model; Minitab 15) with time (days after invertebrate addition) as a covariate; data not meeting assumptions of linearity were log-transformed if necessary. Significant ($P \leq 0.05$) time*treatment interaction effects were investigated further using one-way Analysis of Variance (ANOVA) and Tukey's pairwise comparison on extension rates.

Hyphal coverage and fractal dimension data were non-linear and analysed using Repeated Measures ANOVA (RMANOVA; SPSS, Release 16) with treatment as the main effect and time as sub-factor. Treatment data met the assumptions of RMANOVA, being normally distributed (Kolmogorov-Smirnov Test) and with equal variance (Levene's Test). Huynh-Feldt adjusted degrees of freedom and *P*-values were used where sphericity was not assumed (Mauchly's Test of Sphericity). Significant time*treatment interactions were investigated further using one-way ANOVA and Tukey tests on individual time points.

Wood decay rates and final invertebrate numbers were compared across treatments using one-way ANOVA, and Tukey tests when data were normally distributed (Anderson-Darling Test) and variances equal (Levene's Test). Where final invertebrate population data violated assumptions of ANOVA, a Kruskal-Wallis test was used.

3.4 Results

3.4.1 Radial extension

Mycelial extension of all fungi changed linearly with time. Only macro-invertebrates affected mycelial extension of any fungus (Fig. 3.1). Extension rates of *P. velutina* were lower during *B. guttulatus* ($F_{1,36} = 14.13$, $P = 0.001$) and *O. asellus* ($F_{1,36} = 11.61$, $P = 0.002$) grazing than in ungrazed controls; there was no significant difference ($F_{1,36} = 0.17$, $P = 0.678$) between extension rates in these two treatments. *Blaniulus guttulatus* and *O. asellus* reduced extension rates of *H. fasciculare* JH ($P < 0.001$ in both treatments) but only *B. guttulatus* prevented extension of *H. fasciculare* DD2 ($F_{1,56} = 69.24$, $P < 0.001$) and *H. fasciculare* DD3 ($F_{1,56} = 94.25$, $P < 0.001$) (Fig. 3.1). *Oniscus asellus* and *P. scaber* reduced the length of *R. bicolor* cords ($P<0.001$ in both comparisons), removing entire systems in most cases. *Blaniulus guttulatus* also reduced extension rate of *R. bicolor* ($F_{1,46} = 21.85$, $P = 0.002$), but not to the same extent as the two woodlice. No invertebrate significantly ($P \leq 0.05$) affected extension rates of *P. impudicus* (Fig. 3.1).

3.4.2 Hyphal coverage

Panagrellus redivivus, F. candida, P. scaber, O. asellus and *B. guttulatus* affected hyphal coverage of some fungal strains. *Panagrellus redivivus* was the only species that significantly reduced hyphal coverage of *P. velutina* ($F_{8.504,68.034} = 14.13$, $P < 0.001$) and *P. impudicus* ($F_{1.816,14.524} = 5.186$, $P = 0.022$) (Fig. 3.2). *Resinicium bicolor* coverage was significantly ($P \leq 0.05$) affected by *F. candida, O. asellus, P. scaber* and *B. guttulatus* ($P < 0.001$ in all cases). There was no significant difference between *R. bicolor* coverage in *O. asellus* and *P. scaber,* or *F. candida* and *B. guttulatus* treatments ($F_{3.022,24.175} = 1.154$, $P = 0.348$ and $F_{2.532,20.258} = 3.797$, $P = 0.032$, respectively) (Fig. 3.2). The two woodlouse species consumed entire *R. bicolor* systems, while *F. candida* and *B. guttulatus* only consumed small areas of mycelia within larger networks (Fig. 3.2).

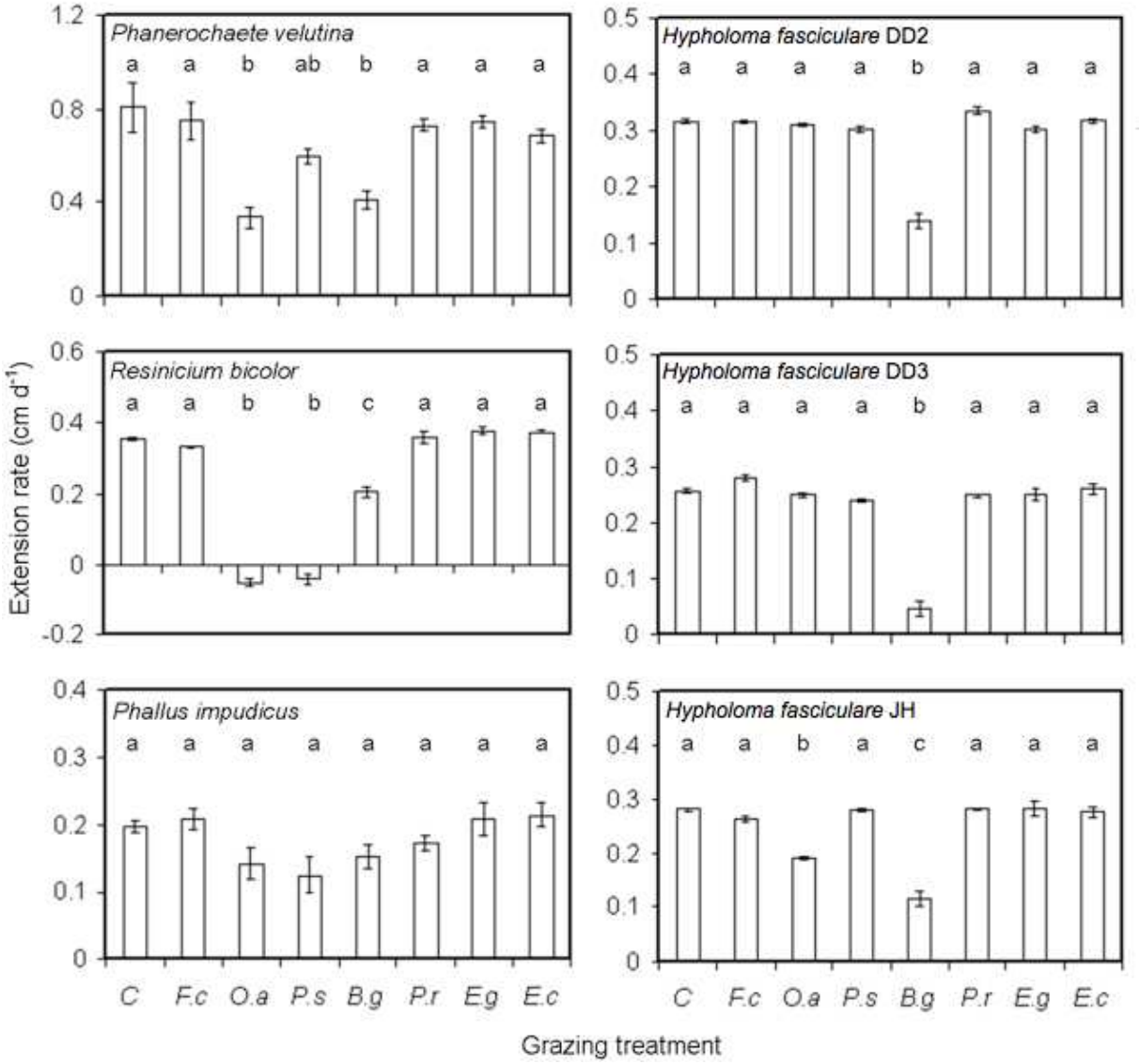

Fig. 3.1: Radial extension rates (mean ± standard error) of *Phanerochaete velutina*, *Resinicium bicolor*, *Phallus impudicus*, *Hypholoma fasciculare* DD2, *Hypholoma fasciculare* DD3, *Hypholoma fasciculare* JH growing across compressed non-sterile soil from a 2 cm^3 beech wood block during fungus-only control (*C*), *Folsomia candida* (*F.c*), *Oniscus asellus* (*O.a*), *Porcellio scaber* (*P.s*), *Blaniulus guttulatus* (*B.g*), *Panagrellus redivivus* (*P.r*), *Euzetes globulus* (*E.g*) or *Enchytraeus crypticus* (*E.c*) grazing treatments. Different letters above bars indicate significant differences in extension rates (ANCOVA; $P \leq 0.05$); y-axis scales vary between graphs.

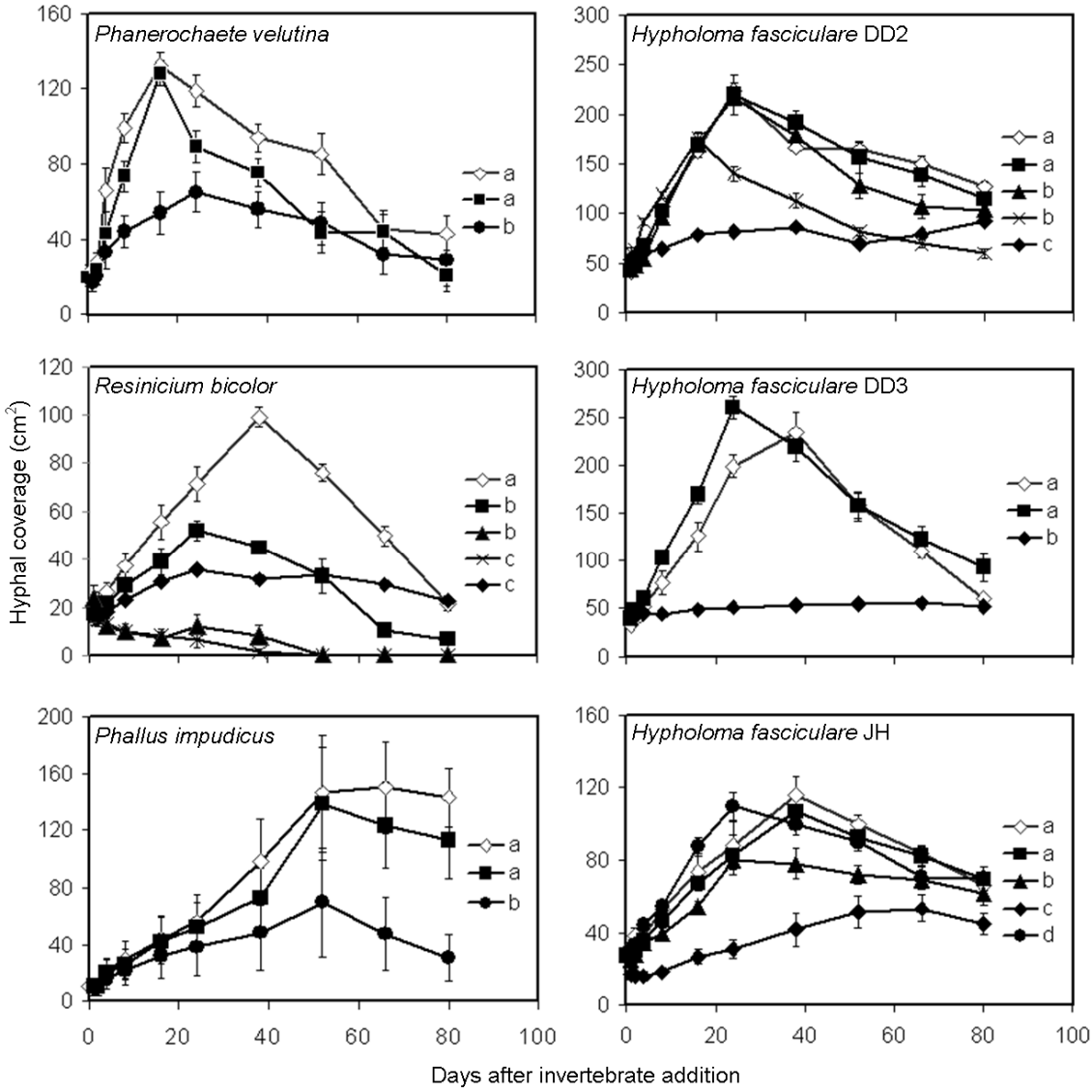

Fig. 3.2: Hyphal coverage of *Phanerochaete velutina*, *Resinicium bicolor*, *Phallus impudicus*, *Hypholoma fasciculare* DD2, *Hypholoma fasciculare* DD3, *Hypholoma fasciculare* JH, over 80 d during fungus-only control (◇), *Folsomia candida* (■), *Oniscus asellus* (▲), *Porcellio scaber* (**X**), *Blaniulus guttulatus* (◆) or *Panagrellus redivivus* (●) grazing treatments. y-axis scales vary between graphs. Different letters in the legend indicate significant differences in hyphal coverage between treatments over time (Repeated Measures ANOVA; $P \leq 0.05$). For clarity, not all grazing treatments are included; treatments not significantly different from the controls are not shown.

The three strains of *H. fasciculare* were differentially affected by invertebrate grazers. All three strains were significantly ($P < 0.001$) reduced by *B. guttulatus* at both 28 (when mycelia reached tray edges) and 80 d (Fig. 3.2). No other invertebrate reduced hyphal coverage of any *H. fasciculare* strain before mycelia reached the tray edges, although *F. candida* grazing

resulted in increased coverage of *H. fasciculare* DD3 by 24 d ($F_{3.312,26.497}$ = 87.215, P < 0.001). Over 80 d *H. fasciculare* DD2 was significantly (P ≤ 0.05) affected by both *O. asellus* ($F_{7.222,57.778}$ = 4.264, P = 0.001) and *P. scaber* ($F_{6.235,49.879}$ = 22.942, P < 0.001), and no other species affected hyphal coverage of *H. fasciculare* DD3. *Hypholoma fasciculare* JH coverage was reduced by *O. asellus* ($F_{6.729,53.829}$=4.59, P =0.001) and increased during P. redivivus ($F_{5.549,44.388}$ = 3.662, P = 0.006) grazing treatments over 80 d (Fig. 3.2).

3.4.3 Fractal dimensions

RMANOVA of fractal dimension (D_{BM}) confirmed that invertebrates had species-specific impacts on morphology of all six fungi (*P. velutina*: $F_{19.952,91.239}$ = 8.69, P < 0.001; *R. bicolor*: $F_{18.015,84.070}$ = 2.443, P = 0.003; *P. impudicus*: $F_{11.786,55}$ = 2.076, P = 0.035; *H. fasciculare* DD2: $F_{23.088,107.744}$ = 16.703, P < 0.001, DD3: $F_{21.741,101.458}$ = 6.225, P < 0.001 and JH: $F_{19.519,91.087}$ = 3.728, P < 0.001). Fractal dimension mirrored hyphal coverage in all treatments, except that both *E. globulus* and *E. crypticus* increased D_{BM} from that of controls in *H. fasciculare* JH (P = 0.013 and P = 0.004, respectively).

3.4.4 Feeding strategies

Millipede and woodlouse species preferentially grazed thick mycelial cords (Fig. 3.3; A2; B2). Grazing by the latter was concentrated on the thick cords of *R. bicolor*. The surrounding hyphae were ignored during the early stages of grazing (Fig. 3.3E). *B. guttulatus* behaved in a similar way whilst grazing all fungal resources. The millipedes, by positioning themselves on un-colonised soil and facing growing mycelial tips, were able to pivot the front half of their bodies and make crescent-shaped indentations in the growing fronts of fungal networks. This was most apparent in fungi with a diffuse growing front such as *H. fasciculare* DD2 (Fig. 3.3; A2). Millipede grazing prevented mycelial extension beyond the point of grazing.

F. candida and *E. globulus* generally fed on the fine white hyphae of *R. bicolor* (Fig. 3.3; G, H). As a result, *F. candida* grazing reduced hyphal coverage and fractal dimension of *R. bicolor* without affecting extension rates of thick cords. *E. crypticus* fed solely on *R. bicolor,* disrupting mycelia at the base of the wood block (Fig. 3.3; I). This damage was not sufficient to have any significant effects on mycelial development.

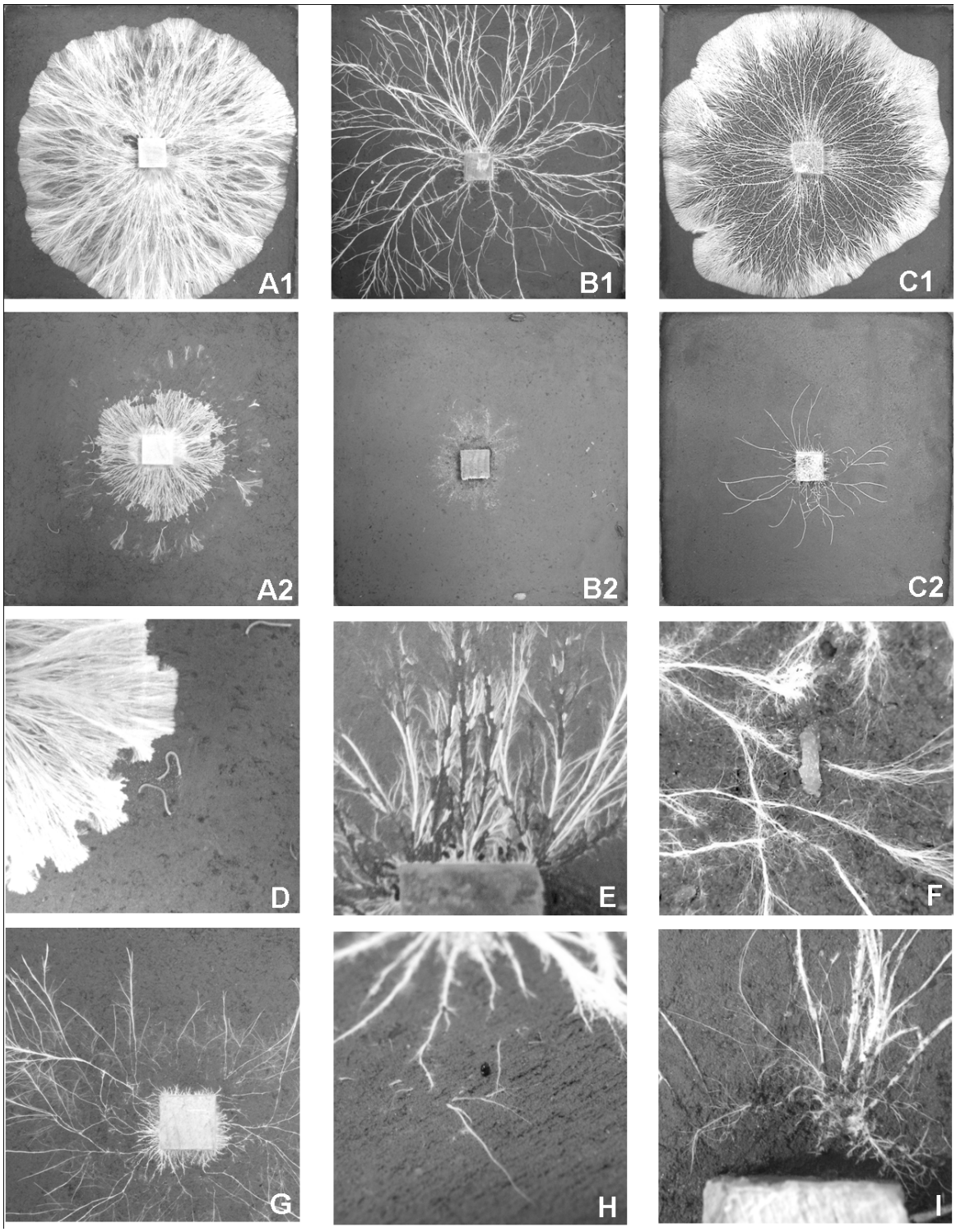

Fig. 3.3: Digital images showing un-grazed control growth of *Hypholoma fasciculare* DD2 (A1), *Resinicium bicolor* (B1) and *Phanerochaete velutina* (C1), and grazing impacts of *Blaniulus guttulatus* (A2), *Oniscus asellus* (B2) and *Folsomia candida* (C2), respectively, after 10 d of growth from 2 x 2 x 1 cm wood blocks on 24 x 24 cm soil trays. Also shown are grazing styles of *B. guttulatus* on *H. fasciculare* (D), *Porcellio scaber* on *R. bicolor* (E), *P. redivivus* on *P. velutina* (F), *Folsomia candida* (G), *Euzetes globulus* (H) and *Enchytraeus crypticus* on *R. bicolor* (I).

Panagrellus redivivus was the only species tested using a stylet to penetrate mycelial hyphae and gain access to fungal cell contents. This feeding caused the gradual regression of entire mycelia (Fig. 3.3) which contrasts with the direct removal by other grazer species. *Panagrellus redivivus* also showed an aggregating behaviour, forming clumps of 50-100 individuals 0.2 - 1 cm in diameter.

3.4.5 Wood decay rates

Decay rates of beech wood varied between fungal species and strains. *Hypholoma fasciculare* DD3 and *H. fasciculare* JH decomposed wood at a slower rate than *P. velutina* and *R. bicolor* ($F_{1,8} = 6.71$, $P = 0.032$), but at a faster rate than *H. fasciculare* DD2 and *P. impudicus* ($F_{1,8} = 18.48$, $P = 0.003$) (Fig 3.4). Decay rates also varied between invertebrate treatments. Wood inoculated with *R. bicolor* decayed at a significantly ($F_{7,32} = 1.621$, $P = 0.0418$) faster rate during *O. asellus* grazing than in ungrazed control treatments (Fig. 3.4). Decay rates of *P. velutina* wood blocks also increased during *O. asellus* ($F_{7,32} = 3.04$, $P = 0.011$) and *P. redivivus* ($F_{7,32} = 3.04$, $P<0.001$) grazing, while *B. guttulatus* grazing increased decay rates of wood blocks inoculated with all three *H. fasciculare* strains (DD2: $F_{7,32} = 3.477$, $P=0.002$; DD3: $F_{7,32} = 3.022$, $P=0.005$; JH: $F_{7,32} = 1.953$, $P=0.005$).

3.4.6 Final invertebrate numbers

Population numbers of *F. candida, O. asellus* and *P. scaber* were all reduced on *H. fasciculare* strains*, P. velutina* and *P. impudicus,* compared to those in invertebrate-only controls. Only on *R. bicolor* were populations higher than on the invertebrate-only controls (*F. candida*: $P = 0.004$; *O. asellus*: $P = 0.01$; *P. scaber*: $P = 0.009$) (Table 3.2). *Euzetes globulus* populations were also significantly ($P \leq 0.05$) reduced by all *H. fasciculare* strains and *P. impudicus,* but were not significantly ($P > 0.05$) different from invertebrate-only controls when feeding on *R. bicolor* ($F_{1,8} = 0.01$, $P = 0.94$) or *P. velutina* ($F_{1,8} = 1.98$, $P = 0.197$). All *B. guttulatus* individuals in control trays had died by 80 d. There were no significant ($P > 0.05$) differences between final populations in the different fungal treatments, though all were significantly ($P \leq 0.05$) higher than on controls. *Panagrellus redivivus* populations were reduced on *R. bicolor* and all three *H. fasciculare* strains*,* but were not significantly ($P > 0.05$) different from controls when feeding on *P. velutina* or *P. impudicus.* *Enchytraeus crypticus* populations only survived in *R. bicolor* treatments, being reduced to zero on all other fungi and control trays.

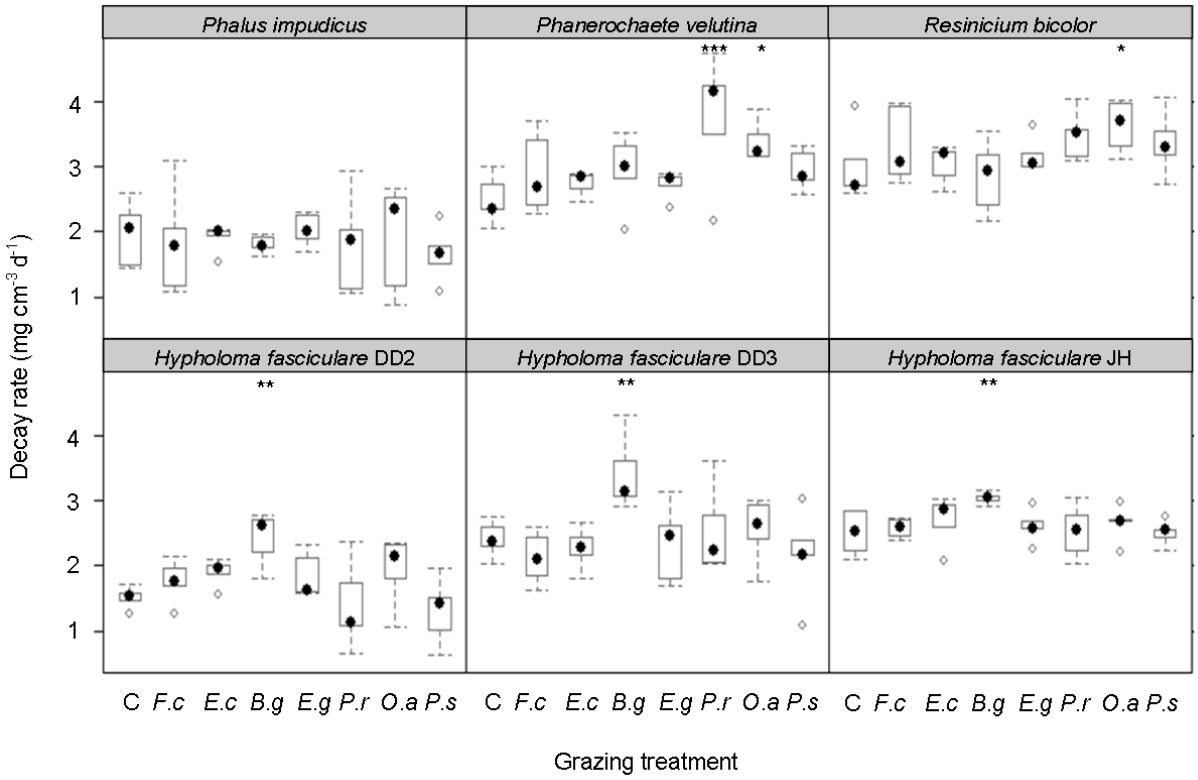

Fig. 3.4: Decay rates (mg cm^{-3}) of beech (*Fagus sylvatica*) wood blocks colonised by *Phallus impudicus*, *Phanerochaete velutina*, *Resinicium bicolor* and *Hypholoma fasciculare* strains DD2, DD3 and JH during control (C), *Folsomia candida* (F.c) *Enchytraeus crypticus* (E.c), *Blaniulus guttulatus* (B.g), *Euzetes globulus* (E.g), *Panagrellus redivivus* (P.r), *Oniscus asellus* (O.a) and *Porcellio scaber* (P.s) grazing treatments. Black dots indicate mean decay rates of five replicates of each treatment, boxes represent interquartile ranges and dotted lines indicate the range. Open circles represent treatment outliers. Asterisks indicate significant differences (ANOVA; $P \leq 0.05$) from un-grazed controls (***: P < 0.001, **: P < 0.01, *: P <0.05).

3.5 Discussion

Soil-inhabiting invertebrate taxa differentially affect the ability of basidiomycete fungi to forage for, and decompose wood resources. Taxon-specific grazing effects have implications for fungal decomposition, nutrient cycling and carbon storage in woodland ecosystems (Hättenschwiler *et al.* 2005; Gessner *et al.* 2010). Grazing effects vary markedly. For example, the nematode *P. redivivus* increased hyphal coverage of *H. fasciculare* JH mycelia, while heavy millipede, *B. guttulatus,* grazing prevented growth and extension of the same

Table 3.2: Numbers (mean ± standard error of the mean) of invertebrates after 80 d in microcosms. Different letters in rows indicate significantly (ANOVA; $P \leq 0.05$) different final populations feeding on different fungi. – indicates not significantly different from invertebrate-only controls.

	Control	*Phanerochaete velutina*	*Resinicium bicolor*	*Phallus impudicus*	*Hypholoma fasciculare* DD2	*Hypholoma fasciculare* DD3	*Hypholoma fasciculare* JH
Oniscus asellus	2 ± 0.3 a	0.2 ± 0.2 b	4 ± 0.3 c	0.2 ± 0.2 b	0.4 ± 0.4 b	0.2 ± 0.2 b	0.4 ± 0.4 b
Porcellio scaber	1.6 ± 0.5 a	0.6 ± 0.4 b	2.2 ± 0.4 c	0 ± 0 d	0.6 ± 0.4 b	0.6 ± 0.4 b	1 ± 0.3 b
Blaniulus guttulatus	0 ± 0 a	1.4 ± 0.24 b	1.2 ± 0.58 b	0.4 ± 0.4 b	2.2 ± 0.8 b	2.6 ± 0.5 b	2.4 ± 0.5 b
Folsomia candida	643 ± 20 a	368 ± 64.8 b	1371.8 ± 174.6 c	_	17.4 ± 7.6 d	22.8 ± 17.6 d	7.8 ± 5 5 d
Euzetes globulus	31 ± 3.6 a	_	_	10.6 ± 3.8 b	11 ± 3.7 b	10.6 ± 2.6 b	17.8 ± 5.3 b
Enchytraeus crypticus	0 ± 0 a	_	43.2 ± 9.56 b	_	_	_	_
Panagrellus redivivus	3700 ± 538.5 a	_	1200 ± 561.2 b	_	100 ± 100 b	200 ± 122.4 b	400 ± 187.4 b

fungal species. The stimulated growth during nematode activity, recorded previously in different *H. fasciculare* strains and *P. velutina* in response to collembola grazing (Tordoff *et al.* 2006; Bretherton *et al.* 2006), is analogous to the compensatory growth response observed in plants after herbivory (McNaughton 1983; Hedlund *et al.* 1991). This morphological response to grazing was characterised by increased hyphal branching (fractal dimension) and promoted space-filling by foraging mycelia. Grazing by nematodes and collembola will, thus, influence the ability of fungi to forage for and obtain nutrients from the soil while the destructive impacts of macrofauna (*O. asellus, P. scaber* and *B. guttulatus*) delaying, or even preventing, mycelial extension will influence the rates at which basidiomycete systems encounter and colonise new wood resources.

Grazing impacts were also species-specific within the same invertebrate order. Although both woodlouse species yielded many similar results (e.g. both consumed entire *R. bicolor* systems and had no effects on *P. impudicus*), there were some marked differences. *Oniscus asellus* reduced extension of *P. velutina* and *H. fasciculare* JH, while *P. scaber* had no effect. This may be explained by subtle interspecific differences in gut physiology (Hopkin 1990) that

may have led to variation in feeding behaviour. Species-specific effects on basidiomycete mycelia have also been reported in collembola; the metabolic demands of the highly fecund *F. candida* caused greater grazing of basidiomycete mycelia than by the less active *Protaphorura armata* (Tordoff *et al.* 2008).

Invertebrate grazing potentials depend on a range of factors including morphology, life strategy and feeding preferences. Exploring these may enable identification of functional group patterns among soil fauna in their effects on fungal mycelia. For example, *P. scaber, O. asellus* (woodlice) and *B. guttulatus* (millipede) were the only species which reduced extension rates of basidiomycete cords. Their larger mandible and body size may have enabled macrofauna to sever thick cords inaccessible to smaller species. To my knowledge, this is the first report of direct grazing by Isopoda or Myriapoda on basidiomycete mycelia and concurs with Bradford *et al.* (2002) who highlighted the potential importance of macrofauna in determining mycelial functioning. Even between species of comparable size, differences occur. For example, the two mesofaunal species, *F. candida* and *E. globulus,* both have the capacity to affect *R. bicolor* hyphal coverage (A'Bear *et al.* 2010), but in the present study, *F. candida* populations caused significantly more hyphal damage. This difference may be related to life-strategy (Kaneko *et al.* 1998). *F. candida* reproduce rapidly using parthenogenesis (final populations reaching approximately 1370 by 80 d) while the more K-selected *E. globulus* only reached a maximum of 31 individuals whilst grazing *R. bicolor*. These two populations exerted very different grazing pressures on foraging basidiomycetes.

Feeding hierarchies (or preferences) are also common among mycophagous soil fauna (Newell 1984b; Klironomos *et al.* 1992; Maraun *et al.* 2003); to date, most invertebrates have shown preferences for similar fungal resources (e.g. dark pigmented fungi; see Maraun *et al.* 2003). This, however, was not apparent in the present study. *Porcellio scaber,* for example, grazed *R. bicolor* to a greater extent than it consumed any other fungus, while *B. guttulatus* restricted grazing almost entirely to *H. fasciculare.* Taxon-specific selection of fungi by soil invertebrates may be indicative of different nutrient requirements or abilities to withstand fungal toxins (Hiol Hiol *et al.* 1994). The nematode, *P. redivivus*, was the only species to reduce hyphal coverage of *P. impudicus* and *P. velutina*. Both these fungi synthesize sesquiterpenes (Hynes *et al.* 2007); produced in the cell walls, these secondary metabolites are likely to be used in defence against fungivores (Ladygina *et al.* 2006; Kempken & Rohlfs 2010). The use of a penetrating feeding stylet may have enabled nematodes to attain hyphal

cell contents without ingesting cell walls (Yeates *et al.* 1993), avoiding the associated secondary metabolites. The clumping of *P. redivivus* (Fig. 3.3, F) may also enable the nematodes to withstand the harsh environments created by *P. impudicus* and *P. velutina*; individuals in the centre of the clump are shielded from fungal toxins and digestive enzymes (Croll 1970). The reported lack of specialisation within the huge diversity of soil fauna remains one of the 'enigmas of soil ecology' (Maraun *et al.* 2003; Setälä *et al.* 2005). Taxon-specific selective grazing, as reported in this study, may indicate a form of resource partitioning which permits the co-existence of decomposer species within a single habitat.

Fungal species and strains also differ in their susceptibility to grazing. Fungal susceptibility is linked to palatability and is probably influenced by mycelial texture and morphology (Tordoff *et al.* 2006; Bretherton *et al.* 2006). Collembola are known to graze juvenile hyphae more readily than thickened, mature mycelia (Wiggins *et al.* 1979; Hiol Hiol *et al.* 1994). In the present study, negative grazing effects of micro- and mesofauna were only apparent on fungal systems characterised by thin, loosely-aggregated mycelia; extra space around mycelial cords probably making these fungal morphotypes more accessible to grazers (Tordoff *et al.* 2006). Intraspecific differences between *H. fasciculare* strains provide further support to this hypothesis with *P. scaber* reducing hyphal coverage in the sparsely distributed cords of strain *H. fasciculare* JH but having no impact on the dense mycelial mat of strains *H. fasciculare* DD2 or DD3. The diffuse growth of *H. fasciculare* DD2 and DD3 also allowed them to cover a greater surface area of soil than the other fungi. The impacts of grazing may have been relatively less destructive for these larger mycelial systems. Reduced survival of invertebrates feeding on less favourable resources (Table 3.2) suggests that fungi may also have biochemical or metabolic features that influence feeding (Kempken & Rohlfs 2010). As well as sesquiterpenes, oxalic acid, a by-product of lignin decomposition, is also produced by basidiomycete fungi under unfavourable conditions such as during invertebrate attack (Shimada *et al.* 1997). This is precipitated as an insoluble salt, calcium oxalate (Dutton *et al.* 1993), and may, as in many plant species (Molano-Flores 2001), deter invertebrate grazers. Variation in fungal morphology and biochemistry indicates that grazing undoubtedly acts as a selective pressure favouring unpalatable fungi, and influencing community composition of basidiomycetes within woodland soils.

The indirect effects of fungus-invertebrate interactions were apparent in the increased decay of wood blocks during heavy grazing. Growth responses of basidiomycetes to grazing are

mediated by increased enzyme production and nutrient uptake from woody resources (Hedlund *et al.* 1991). The increased rate of wood decay during heavy grazing (*c.f.* collembola grazing in Tordoff *et al.* 2008) is a result of such enzyme activity and adds further support to the stimulatory affects of saprophagous macrofauna on fungus-mediated decomposition (Bradford *et al.* 2002; Hättenschwiler *et al.* 2005). Changes to fungal community composition, brought about by selective grazing, may also affect rates of woody decay as a consequence of shifting enzymatic capabilities (Newell 1984b; Gessner *et al.* 2010). Differences between the decay rates of fungi in the present study suggest that grazer-induced shifts in fungal community composition could significantly alter the rates of decomposition and nutrient turnover.

3.6 Conclusions

Invertebrate populations differentially affect mycelial growth, foraging and activity of basidiomycete fungi. Selective grazing by invertebrate taxa could strongly influence fungal communities. Invertebrate functional groups, determined by morphology, physiology and life-strategy, determine interaction consequences and a detailed characterisation of these groups may provide compelling predictors of the effects of changing soil biodiversity on woodland decomposition (Gessner *et al.* 2010). Grazer-induced increases in wood decay rates further highlight the significance of these interactions for soil ecosystem functioning. While it is clear that climate change drivers such as elevated moisture or CO_2 affect fungal activity directly (Gange *et al.* 2007) this study suggests that the predicted changes in invertebrate community composition (Jones *et al.* 1998; Wolters *et al.* 2000; Bokhorst *et al.* 2008) will also alter patterns of mycelial growth and functioning within soils. Changing invertebrate communities may also alter the selective pressures on fungal community compositions. This has implications for nutrient cycling, carbon sequestration and productivity in soils under current and future climatic scenarios (Loreau *et al.* 2001; Wardle *et al.* 2004; Hättenschwiler *et al.* 2005).

4. Species-specific effects of grazing invertebrates on mycelial emergence and growth from woody resources into soil

4.1 Abstract

Extensive studies on the grazing of young basidiomycete mycelial systems by invertebrates have revealed effects on extension rate, hyphal coverage and fractal geometry. To date, no studies have compared the grazing effects of different invertebrates on the ability of fungi to emerge from wood and establish mycelial systems in soil. Here, the effects of six soil invertebrate taxa on mycelial emergence and subsequent development of six basidiomycetes were compared. Woodlouse (*Oniscus asellus*), millipede (*Blaniulus guttulatus*), oribatid mite (*Euzetes globulus*), collembola (*Folsomia candida*), enchytraeid (*Enchytraeus crypticus*) and nematode (*Panagrellus redivivus*) populations were allowed to graze *Phanerochaete velutina, Resinicium bicolor, Phallus impudicus* and three different isolates of *Hypholoma fasciculare* mycelia as they emerged from beech (*Fagus sylvatica*) wood-inocula in 2-D soil-tray microcosms. Impacts varied between invertebrate taxa, ranging from woodlice, which affected mycelial development of all fungal isolates and completely prevented mycelial growth in two fungal species, to mites and enchytraeids which had no discernable effects on any of the fungi. Grazing impacts also varied between and within fungal species. Wood decay rates were affected with implications for nutrient mineralisation and decomposition.

4.2 Introduction

Saprotrophic basidiomycetes are primarily responsible for wood and leaf litter decomposition in woodland soils. Their biomass and respiration often exceeds that of all other organisms within detritus-based food chains (Osono 2007; Gessner *et al.* 2010). Many basidiomycetes produce dynamic mycelial networks which grow at the soil-litter interface, connecting patchily distributed woody resources, and through which nutrients and water are readily translocated (Boddy 1993; 1999; 2000). Cord persistence and compensatory growth responses indicate a resistance to invertebrate grazing (Hedlund *et al.* 1991; Tordoff *et al*. 2006), but even at low-density, collembola and woodlouse populations can consume entire mycelial systems (Chapter 3). Grazing by invertebrates directly affects the abilities of basidiomycetes to forage for, and decompose, dead organic material (Tordoff *et al*. 2006). As a consequence of selective grazing, microbial community composition may be altered and ecosystem functioning affected (Hättenschwiler *et al.* 2005).

A wide range of soil invertebrate taxa can feed on, and affect spatial distribution of, cord-forming basidiomycetes (Chapter 3). Invertebrates have species-specific impacts on fungal mycelia; different faunal grazing strategies and preferences have different implications for mycelial growth and morphology. Until now, the majority of microcosm-based grazing interactions have been studied using mycelial networks already established on the soil surface (Kampichler *et al.* 2004; Harold *et al* 2005; Rotheray *et al.* 2009). The abundance of soil invertebrates (Giller 1996; Maraun *et al.* 2003), and the tendency of many taxa (e.g., woodlice and millipedes) to live in or under decaying wood (Edney 1954; Brookes & Willoughby 1978), however, suggests that grazing may often occur immediately upon mycelial emergence from woody resources. This is an important phase of fungal development as it is not until mycelial cords have become established that basidiomycete networks can persist in woodland soils (Boddy 1993; 1999). The potential of fungi to develop and form mycelial networks may, thus, depend on their ability to withstand or avoid grazers during emergence. To date, only one study (Tordoff *et al.* 2006) has addressed this; showing that the collembola, *Folsomia candida,* delayed cord formation of *Hypholoma fasciculare* and *Resinicium bicolor*. The potential of grazing invertebrates, other than collembola, to affect this limiting step in basidiomycete development is unknown.

When introduced onto established fungal networks, woodlice (*Oniscus asellus* and *Porcellio scaber*) and millipedes (*Blaniulus guttulatus*) prevented mycelial extension of *R. bicolor* and *H. fasciculare,* respectively (Chapter 3). This suggests that macroinvertebrates have the capacity not only to affect mycelial development, but also prevent outgrowth entirely. While grazing by meso- and micro-invertebrates affected hyphal coverage (by grazing fine hyphae within mycelial systems) none affected mycelial extension of any fungus (Chapter 3). Grazing is, however, likely to be particularly disruptive to the growth of young, emerging hyphae (Hiol Hiol *et al.* 1994). Wood decay rates by specific fungi can also be affected by intense grazing (Tordoff *et al.* 2006). The present study investigates the effects of grazing invertebrates by six phyla on outgrowth and establishment of four basidiomycete species growing from wood blocks in soil microcosms.

The selected invertebrate species were among the most common representatives of their respective orders (Isopoda, Julida (Class Diplopoda), Oribatida, Collembola, Tubificida and Rhabditida (Phylum Nematoda)) found in local woodland soil (Coed Beddick Inclosure, Tintern, see Chapter 3 for location details) and represented micro-, meso- and macrofauna species. The six species covered a range of different feeding mechanisms; chewing mandibles and maxillae of different sizes were represented in most macro- and mesofauna, while the nematodes utilised penetrating stylets to access hyphal contents. All fungal species are common in temperate woodlands and likely to co-occur with each other and the invertebrates. Different genetic isolates of *Hypholoma fasciculare* allowed investigation of both inter- and intraspecific responses to grazing. Changes to mycelial extension, hyphal coverage and fractal dimensions were compared over time between invertebrate treatments for each fungal strain. It was predicted that: (i) invertebrates would have taxon-specific impacts on mycelial development; (ii) grazing impacts would differ from those on pre-established systems; (iii) macro-fauna populations would have the greatest impacts on outgrowth and cord formation; and (iv) heavy grazing would increase wood decay rates by fungi as increased nutrient uptake is necessary to counteract the negative effects of grazing as in Tordoff *et al.* (2006).

4.3 Materials and methods

4.3.1 Fungal culturing and inoculation

Hypholoma fasciculare (three strains: DD2, DD3 and JH)*, Resinicium bicolor, Phanerochaete velutina* and *Phallus impudicus* (Cardiff University Culture Collection) were routinely cultured in non-vented 9 cm diam. Petri dishes on 2% malt extract agar (MEA; 20 g^{-1} Munton & Fiston malt, 15 g^{-1} Lab M agar No. 2). Freshly-felled beech wood (*Fagus sylvatica*) was cut into blocks (2 x 2 x 1 cm) and frozen at -18°C until required. Wood blocks were thawed in deionised water (DH_2O) before being autoclaved at 121°C for 20 min in double, sealed autoclave bags. This process was repeated twice in the 24 hr prior to use. Sterilized wood blocks were added to fungal cultures in 13 cm diam. Petri dishes, sealed with Nescofilm® and incubated for three months at 21°C in the dark.

4.3.2 Invertebrate collection and culturing

Oribatid mites - *Euzetes globulus* (Acari, Oribatida, Euzetidae) (extracted using Tüllgren funnels from soil collected to a depth of 10 cm from deciduous woodland in the Coed Beddick Inclosure), collembola - *Folsomia candida* (Collembola, Isotomidae) (Cardiff University Collembola Culture), millipedes, *B. guttulatus* (Myriapoda, Julida, Blaniulidae (Fabricius 1798)) and woodlice - *Oniscus asellus* (Isopoda, Oniscidae) (collected from Coopers Field, Bute Park, Cardiff, see Chapter 3 for location details) were cultured in 0.6 l culture pots on a medium of 90% plaster of Paris (Minerva Dental, Cardiff, UK) and 10% charcoal (Sigma, Poole, UK). Pots had vented lids and were kept in the dark at 20°C. Cultures were kept moist using DH_2O and fed weekly with dried baker's yeast (Spice of Life, Cardiff, UK).

Nematode, *Panagrellus redivivus* (Rhabditida, Panagrolaimidae) (UK Parasitology Group, Aberystwyth University), cultures were maintained in a mixture of 35 g porridge oats and 60 ml deionised water (DH_2O). The porridge mixture was autoclaved (121°C for 20 min) in 500 ml jars and left to cool for 20 min prior to nematode addition. Enchytraeid, *Enchytraeus crypticus* (Tubificida, Enchytraeidae) (Department of Terrestrial Ecology, National Environmental Research Institute of Denmark), cultures consisted of an agar medium containing 13.6 g Bacti-Agar No. 1, 772 ml DH_2O, 6 ml 0.1 M $NaHCO_3$, 6.4 ml 0.01M KCl, 8 ml $CaCl.2H_2O$ and 8 ml conc. $MgSO_4$. Clean nematode and enchytraeid worm suspensions were obtained using wet funnel extraction (Southwood & Henderson 2000). Worms were

subsequently washed for 60 min in a solution of 5 ppm benomyl and 30 ppm chlorotetracycline to reduce fungal and bacterial contamination, respectively. Cleaned worms were rinsed in DH_2O prior to introduction into microcosms.

The numbers of invertebrates added to soil microcosms were lower than average field densities of each species (Banerjee 1970; Peterson & Luxton 1982; Topp *et al.* 2006). This was done in an attempt to relate densities to a strictly 2-D, rather than field 3-D, environment (Table 3.1). Meso- and macrofauna species were size-selected (Table 3.1) and individually introduced to microcosms. Wet funnel extraction of cleaned nematodes (Chapeter 3) gave an inoculum of 1000 worms, which was applied in 2 ml DH_2O.

4.3.3 Preparation and inoculation of soil trays

Soil was collected to a depth of 20 cm from deciduous woodland in Coed Beddick Inclosure and sieved on site through a 10 mm mesh. Soil was then air-dried, sieved through a 2 mm mesh and frozen at -18°C. Soil was re-wetted with 340 ml DH_2O^{-1} (per kg soil) to attain a soil matric potential of -0.012 MPa prior to use. Wet soil (200 g) was added to 24 x 24 cm diam. bioassay dishes, smoothed and compacted to a depth of 5 mm. Wood blocks cleaned of surface mycelia by a spatula were then centrally placed onto soil trays. Invertebrates were introduced evenly across the soil.

Preparation of 210 trays allowed for five replicates of each interaction and five control trays of each fungus growing with no invertebrate grazers. Trays were stacked and sealed in polythene bags to reduce water loss and incubated at 21˚C and 70 % humidity. Density (dry weight/fresh volume; g cm^{-3}) of five extra wood blocks from each fungal strain was determined at the start of the experiment. At the end of the experiment the process was repeated to determine densities of wood blocks from interaction trays. Decay rate (mg cm^{-3} d^{-1}) was estimated by subtracting final densities from those at the start of the experiment.

4.3.4 Image capture and analysis

Digital images were captured using a Nikon Coolpix 57000 camera, mounted on a camera stand at a height of 40.5 cm after 7, 14, 21, 35, 49, 63 and 77 d. Images were analysed using IMAGEJ (National Institute of Health, USA). A 2 cm line was drawn against a ruler to the side of the trays for calibration. Tray edges and wood blocks were removed by windowing and the resulting image converted into 8-bit, and then to binary with a manually set threshold.

Mycelia and soil were indicated by red and black pixels, respectively, allowing hyphal coverage (cm^2) to be calculated (number of red pixels). Mycelial extension was estimated from the mean length of eight lines drawn from the centre of each wood block (at 45° angles from each other) to hyphal tips. Extension rates were recorded for each fungal strain until mycelia from any replicate reached the tray edge. Mass fractal dimension was determined using the box-counting method to provide a quantative value describing space-filling and branching (Obert *et al.* 1990; Donnelly *et al.* 1999; Boddy & Donnelly 2008). This was performed on the final image of each fungus before mycelia reached tray edges.

4.3.5 Statistical analysis

Analysis of Covariance (ANCOVA; General Linear Model; Minitab Statistical software, Release 15) was used to compare radial extension of fungi across controls and treatments, with time as a covariate. Data not meeting assumptions of linearity were log-transformed. Changes in hyphal coverage were analysed using Repeated Measures Analysis of Variance (RM ANOVA; SPSS, Release 16) with invertebrate species as the main effect and time as sub-factor. All data met the assumptions of RM ANOVA, being normally distributed (Kolmogrov-Smirnov A Test) and with equal variance (Levene's Test). Huynh-Feldt adjusted *P*-values were used where sphericity was not met (Mauchly's Test of Sphericity). Significant time*treatment interactions were investigated further using one-way ANOVA on individual time points.

Wood decay rates and fractal dimensions were compared across treatments using one-way ANOVA and Tukey tests when data were normally distributed (Anderson-Darling Test) with equal variances (Levene's Test). Where final invertebrate population data violated assumptions of ANOVA a non-parametric Kruskal-Wallis Test was used.

4.4 Results

4.4.1 Radial extension

Extension of *P. velutina, R. bicolor* and *P. impudicus* was reduced by *O. asellus* and *F. candida,* but not by any other invertebrate species (Fig. 4.1). *O. asellus* prevented mycelial emergence completely in *R. bicolor* and *P. impudicus* (Fig. 4.1); effects of *F. candida* and *O.*

asellus were not significantly ($P > 0.05$) different on *P. velutina.* Grazers had different impacts on extension of the three *H. fasciculare* strains (Fig. 4.1). *H. fasciculare* DD2 was only significantly ($P \leq 0.05$) affected by *O. asellus* while other strains were more susceptible to grazing. *H. fasciculare* DD3 was significantly ($P \leq 0.05$) reduced by *F. candida* and *B. guttulatus; H. fasciculare* JH extension was significantly ($P \leq 0.05$) reduced by *O. asellus, F. candida, B. guttulatus* and *P. redivivus.* Effects of *O. asellus* and *F. candida* on *H. fasciculare* JH were not significantly different ($P > 0.05$), but were significantly ($P \leq 0.05$) greater than those of *B. guttulatus* and *P. redivivus* (Fig. 4.1).

4.4.2 Hyphal coverage

Like extension, hyphal coverage of *P. velutina, R. bicolor* and *P. impudicus* was significantly ($P \leq 0.05$) reduced by *F. candida* and *O. asellus* grazing (Fig. 4.2). *R. bicolor* and *P. impudicus* experienced the greatest damage with most replicates exhibiting no mycelial growth throughout the entire 80 d of woodlouse or collembola grazing (Fig. 4.2). *P. velutina* was reduced by *P. redivivus* to the same extent as by *F. candida* ($P = 0.128$). *O. asellus* had a significantly ($P \leq 0.05$) different effect to *F. candida* and *P. redivivus* on *P. velutina* ($P < 0.001$); early grazing initially reduced hyphal coverage, but increased invertebrate mortality after 20 d allowed a subsequent increase in hyphal coverage which continued until 80 d (Fig. 4.2). Grazers differed in their effects on the three *H. fasciculare* strains. *O. asellus* and *B. guttulatus* significantly ($P \leq 0.05$) reduced coverage of *H. fasciculare* DD2 to similar extents ($P = 0.414$). *H. fasciculare* JH coverage was significantly ($P \leq 0.05$) reduced by *F. candida, O. asellus* and *B. guttulatus*, with most damage being caused by collembola grazing ($P = 0.007$). *H. fasciculare* DD3 was significantly ($P \leq 0.05$) reduced by *F. candida, O. asellus, B. guttulatus* and *P. redivivus*; collembola and millipedes caused the greatest damage, but did not differ in their effects ($P = 0.632$) (Fig. 4.2).

4.4.3 Fractal dimension

Fractal dimensions of *P. impudicus* and *R. bicolor* mirrored hyphal coverage, both being significantly ($P \leq 0.05$) reduced by *F. candida* and *O. asellus* grazing ($P < 0.001$ in all interactions). Fractal dimensions of *P. velutina* were also reduced by *F. candida* ($P = 0.0017$), but increased during *O. asellus* interactions ($P < 0.001$). Both *O. asellus* and *B. guttulatus*

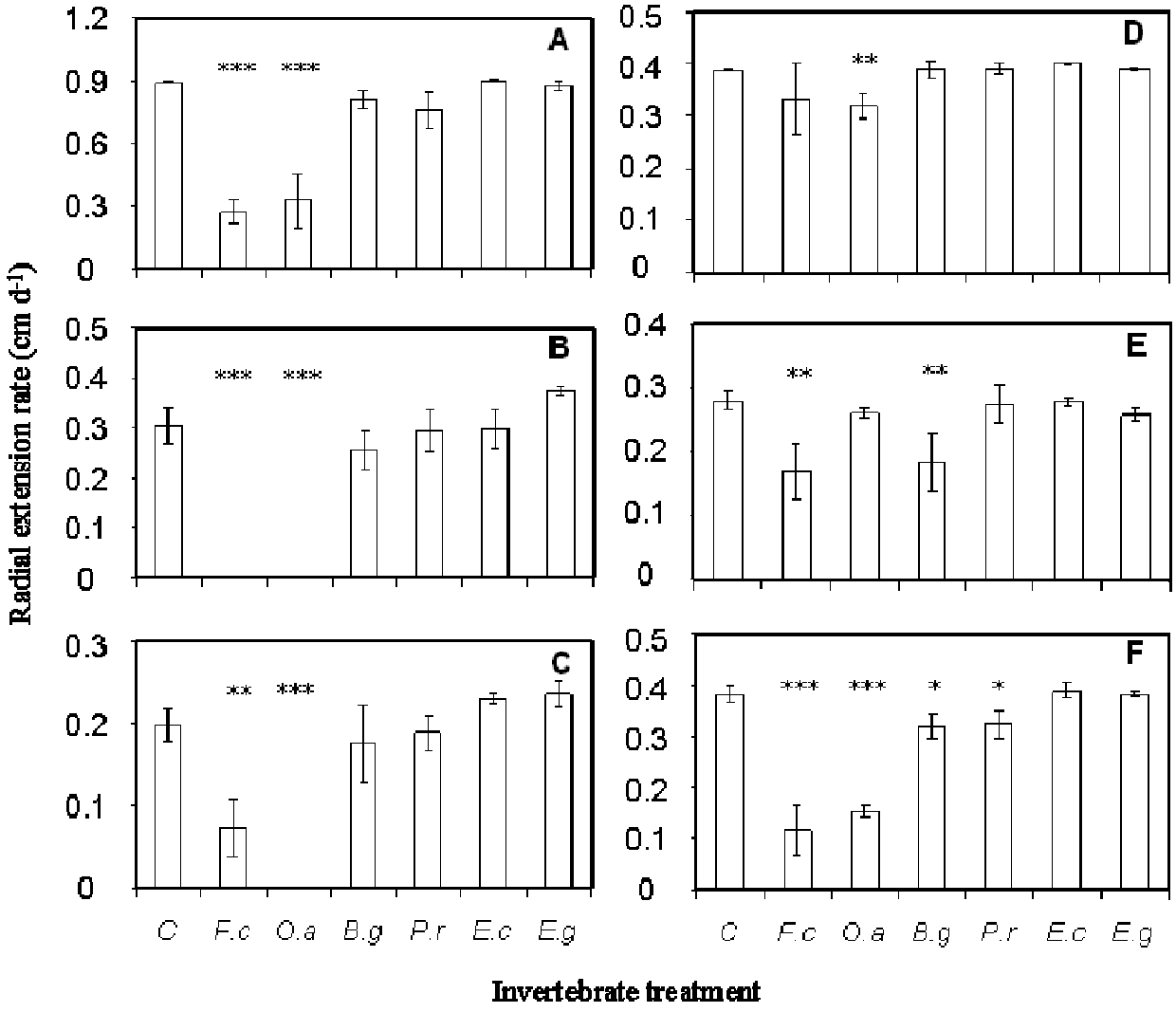

Fig. 4.1: Radial extension rates (mean ± standard error) of: (A) *Phanerochaete velutina*; (B) *Resinicium bicolor*; (C) *Phallus impudicus*; (D) *Hypholoma fasciculare* DD2; (E) *Hypholoma fasciculare* DD3; (F) *Hypholoma fasciculare* JH growing across compressed non-sterile soil from a 2 cm^3 beech wood inocula. Fungus-only control (*C*), *Folsomia candida* (*F.c*), *Oniscus asellus* (*O.a*), *Blaniulus guttulatus* (*B.g*), *Panagrellus redivivus* (*P.r*), *Euzetes globulus* (*E.g*) or *Enchytraeus crypticus* (*E.c*) grazing treatments. Stars indicate significant differences (ANCOVA; $P \leq 0.05$) from un-grazed controls (***: P < 0.001, **: P < 0.01, *: P <0.05). y-axis scales vary between graphs.

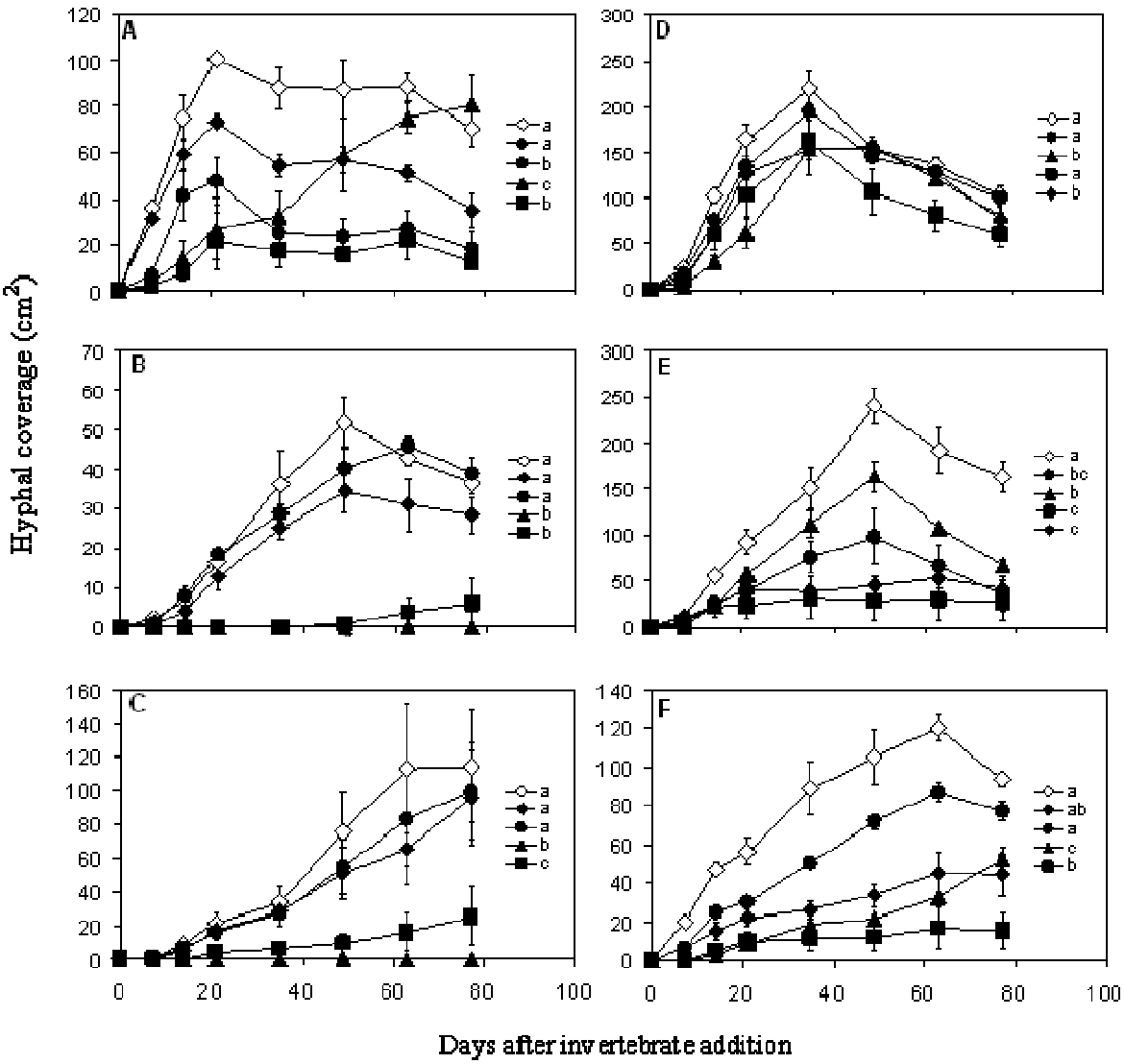

Fig. 4.2: Hyphal coverage of: (A) *Phanerochaete velutina*; (B) *Resinicium bicolor*; (C) *Phallus impudicus*; (D) *Hypholoma fasciculare* DD2; (E) *Hypholoma fasciculare* DD3; (F) *Hypholoma fasciculare* JH, over 80 d in fungus-only control (◇), *Folsomia candida* (■), *Oniscus asellus* (▲), *Porcellio scaber* (**X**), *Blaniulus guttulatus* (◆) or *Panagrellus redivivus* (●) grazing treatments. Scales on y axes vary between fungi. Different letters indicate significant differences in hyphal coverage between treatments over time (Repeated Measures ANOVA; $P \leq 0.05$). Coverage of all fungi during *E. globulus* and *E. crypticus* grazing was not significantly different ($P > 0.05$) from controls and has been omitted for clarity.

grazing reduced branching in *H. fasciculare* DD2 (*O. asellus*: $P < 0.001$ and *B. guttulatus*: $P = 0.004$) and JH ($P < 0.001$ in both interactions), and the latter was also reduced by *F. candida* ($P < 0.001$). *H. fasciculare* branching was reduced by *B. guttulatus* and *F. candida* ($P < 0.001$ in both interactions) and increased by *P. redivivus* ($P = 0.0232$).

4.4.4 Wood block decay rates

Decay rates varied between fungal species but not between *H. fasciculare* strains (Fig. 4.3). Wood blocks colonised by *P. velutina* and *R. bicolor* decayed significantly faster than those colonised by *P. impudicus* ($P = 0.004$) or *H. fasciculare* JH ($P = 0.015$). *H. fasciculare* DD2 and D3 decay rates were intermediate, and not significantly different from any other fungi.

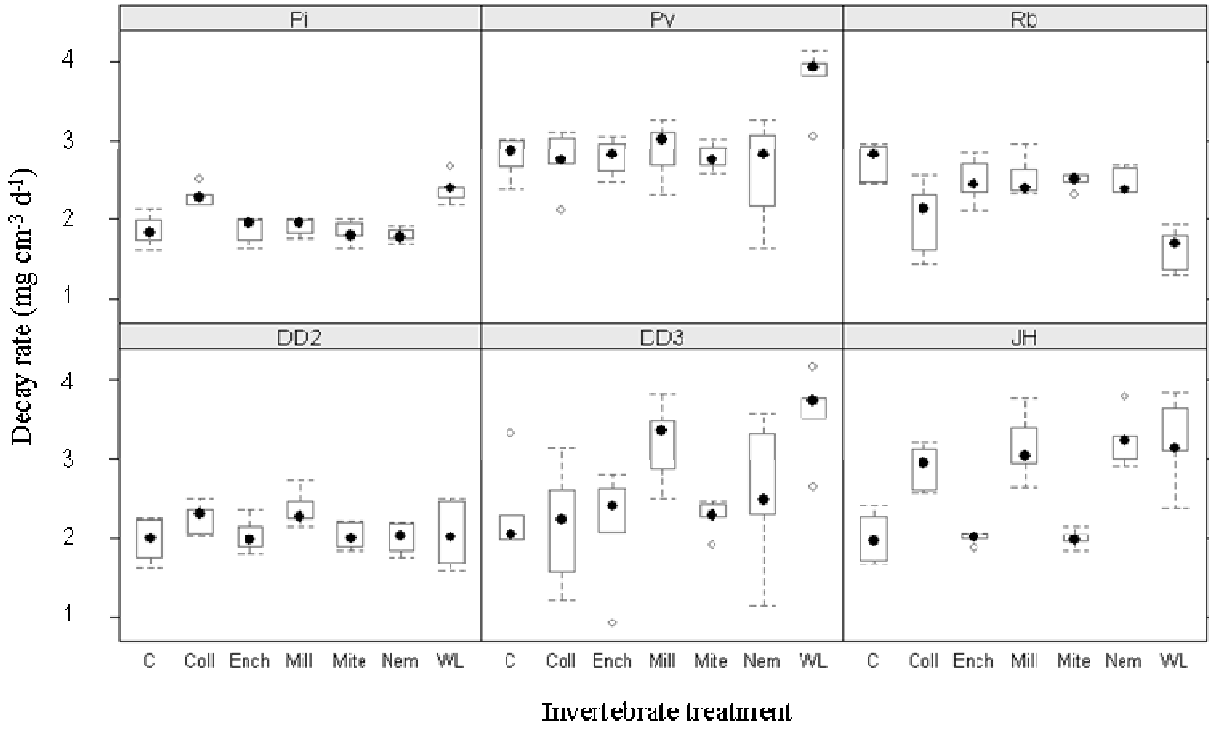

Fig. 4.3: Decay rates (mg cm^{-3} d^{-1}) of beech (*F. sylvatica*) wood blocks colonised by *Phallus impudicus* (Pi), *Phanerochaete velutina* (Pv), *Resinicium bicolor* (Rb) and *Hypholoma fasciculare* (strains DD2, DD3 and JH) during control (C), *Folsomia candida* (Coll) *Enchytraeus crypticus* (Ench), *Blaniulus guttulatus* (Mill), *Euzetes globulus* (Mite), *Panagrellus redivivus* (Nem), and *Oniscus asellus* (WL) grazing treatments. Black dots indicate mean decay rates of five replicates of each treatment, boxes represent inter quartile ranges and dotted lines indicate the range. Open circles represent outliers for each treatment.

Woody decay within fungal strains varied depending on grazing treatments (Fig. 4.3). Decay rates of *P. velutina* wood blocks increased significantly during *O. asellus* grazing ($P = 0.002$). Both *O. asellus* and *F. candida* caused increased decay by *P. impudicus* wood blocks (*O. asellus*: $P = 0.003$; *F. candida*: $P = 0.004$). The same two species had a significant effect on *R. bicolor*, this time reducing decay rates compared with ungrazed controls (*O. asellus*: $P < 0.001$; *F. candida*: $P = 0.016$). Decay rates of *H. fasciculare* DD2 and DD3 increased when grazed by *B. guttulatus* (DD2: $P = 0.037$; DD3: $P = 0.042$). The latter was also increased by *O. asellus* ($P = 0.009$). No other invertebrates affected decay rates of these strains, but *F. candida, O. asellus, B. guttulatus* and *P. redivivus* all increased decay rates of *H. fasciculare* JH (*F. candida*: $P = 0.002$; *O. asellus*: $P = 0.004$; *B. guttulatus*; $P = 0.001$ and *P. redivivus*: $P < 0.001$).

4.5 Discussion

Grazing invertebrates had taxon-specific effects on emergence and subsequent development of mycelial networks. The greatest impacts were associated with woodlouse and collembola populations, the former affecting hyphal coverage in all fungal strains and completely preventing cord extension in two of the four fungal species. Millipede and nematode populations also affected development of some fungi while mites and enchytraeids had no quantifiable impacts on any fungal strains. These invertebrates varied in terms of size, morphology and population dynamics, all of which affect grazing potential (Tordoff *et al.* 2008; Bradford *et al.* 2002). The potential of high intensity grazing to delay, or even prevent mycelial emergence suggests that this may be a particularly vulnerable phase in mycelial development (Boddy 1999). Grazing by soil invertebrates on emerging mycelia will not only determine fungal foraging and nutrient acquisition (Bengtsson *et al*. 1993), but also the existance of extra-resource mycelia in woodland soils. The stark differences between the impacts of invertebrate species suggest that invertebrate species composition is an important factor influencing the establishment and development of mycelial systems in woodland soils.

Grazing impacts also varied between fungi; some species were more resistant to grazing by certain invertebrates. For example, extension rates of *H. fasciculare* DD2 cords were only reduced by woodlouse populations, while extension of *P. impudicus* and *R. bicolor* was

prevented entirely by both woodlouse and collembola grazing. Fungal susceptibility to grazing also varied within species; hyphal coverage of *H. fasciculare* DD3 was reduced by grazing micro-, meso- and macrofauna, while only macro-invertebrates (woodlice and millipedes) influenced *H. fasciculare* DD2 development. Grazing by invertebrate populations will differentially affect the abilities of basidiomycetes to emerge from wood resources and form persistent mycelial networks (Bengtsson *et al*. 1993; Tordoff *et al.* 2006). Varying susceptibility of emerging fungi to grazing may, therefore, be a factor affecting fungal dominance and species composition within soil (Newell 1984a, 1984b).

Fungal susceptibility is linked to palatability (varying with fungal structure, age and physiological status; Moore *et al*., 1985), nutrient content (varies with resource availability and quality; Leonard, 1984) and biochemistry (toxic secondary metabolite synthesis; Parkinson *et al*., 1979). The first two factors will influence the preferences of invertebrates provided with multiple fungal resources. An unpalatable fungus will, however, still be grazed if it is the only available food source, and, in the present study, invertebrates were restricted to individual fungal resources. The reduced grazing of unfavourable fungi is, therefore, the likely result of toxic secondary metabolite production. Calcium oxalate crystals and sesquiterpenes are both produced by basidiomycete fungi under stress conditions such as invertebrate invasion (Shimada *et al.* 1997; Hynes *et al.* 2007). As in plants (Molano-Flores 2001), these secondary metabolites are likely to deter invertebrate grazers. This deterrence will reduce the damage to mycelial systems, but certain fungi are also able to compensate for the grazing disturbance. *Phanerochaete velutina* and *R. bicolor* were both grazed heavily by *O. asellus* during early stages of the interaction (Fig. 4.1), but the accelerated growth of the former enabled it to compensate for this damage and increase in hyphal coverage throughout the experiment. In contrast, *R. bicolor* was removed more rapidly than it was able to extend and was unable to emerge from wood blocks. Variation in fungal biochemistry and growth will, thus, influence the susceptibility of fungi to grazers with implications for soil decomposer communities (Newell 1984a; 1984b). The removal of selected fungi is likely to benefit other microbes competing for similar resources leading to a shift in fungal community composition and activity (Seastedt 1984). Given the importance of fungal diversity in ecosystem functioning and regulation (Mikola *et al*. 2002; Hättenschwiler *et al.* 2005; Gessner *et al.* 2010), understanding the factors affecting the emergence and growth of young basidiomycete mycelia across woodland soil is vital.

The variation in grazing impacts between invertebrate taxa is consistent with a previous study that examined effects of invertebrate grazing on established mycelial systems (Chapter 3). Interaction outcomes were, however, markedly different (Table 4.1). Almost 30% fewer interactions exhibited a significant effect (19 compared to 26) when invertebrates grazed on established mycelial systems. For example, only millipede grazing affected development of established *H. fasciculare* DD3 systems while coverage was reduced by four species when added prior to mycelial emergence. As well as the increased number of significant impacts, the magnitude of most grazing effects were also increased; woodlice and collembola prevented outgrowth of *P. impudicus*, a species which was not affected by either grazer in Chapter 3. These trends were apparent in most fungal strains and suggest that young, fine hyphae emerging from wood resources are likely to be less resilient to grazing than the thick cords present in mature mycelial networks. Preferences of soil collembola for young hyphae have been recorded previously (Anderson & Healey 1972; Wiggins *et al.* 1979); increased rind thickness (Harold *et al.* 2005) or accumulation of calcium oxalate crystals (Connelly & Jellison 1995) would also have reduced the palatability of larger cords. Access to nutrients may also differentially affect the susceptibility of growing systems. Basidiomycetes growing in microcosms, similar to those used in this study but from larger inocula (4 cm^3) have been shown to produce more luxuriant systems, and be less susceptible to *F. candida* grazing, than those growing from smaller resources (Harold *et al.* 2005). Increased uptake of soil nutrients by established mycelial networks in this study may have enabled the production of larger, thicker cords. It may also have allowed earlier synthesis of toxic secondary metabolites and deterrence of invertebrate grazers (Dutton *et al.* 1993; Hynes *et al.* 2007).

The vast abundance of soil invertebrates (Peterson & Luxton 1982; Giller 1996) which are capable of delaying, or preventing mycelial emergence raises the question of how do extensive extra-resource mycelial networks develop in woodland soils? Possibly, basidiomycetes exploit situations where grazer density is low (Tordoff *et al.* 2006). Bretherton *et al.* (2006) showed that extension rates of *P. velutina* increased markedly in grazed systems once collembola grazing ceased. This was also observed in the present study

Table 4.1: The numbers of invertebrate taxa having significant grazing impacts on hyphal coverage, radial extension and wood decay rates in 8 cm diam. established (data from Chapter 3) and non-established (present study) mycelial systems where invertebrates were added prior to mycelial emergence.

	Number of significant grazing impacts					
	Hyphal Coverage		Radial Extension Rate		Wood Decay Rate	
	Established	Non-established	Established	Non-established	Established	Non-established
P. velutina	1	3	1	2	2	1
R. bicolor	3	2	2	2	1	2
P. impudicus	1	2	0	2	1	2
H. fasciculare DD2	2	2	1	1	1	1
H. fasciculare DD3	1	4	1	2	1	2
H. fasciculare JH	3	3	2	2	1	4

during interactions between woodlice and *P. velutina*; heavy grazing, followed by woodlouse mortality, led to increased branching (measured by fractal dimensions) and advanced hyphal coverage by 77 d in grazed rather than ungrazed trays. This over-compensatory and consequent explorative growth (Bretherton *et al.* 2006) may enable the development of the large mycelial systems found throughout temperate woodland soils (Boddy 1999). Persistence of mycelial systems then relies on the efficient uptake of nutrients, accumulation of metabolic by-products (e.g. calcium oxalate crystals) and production of chemical defences, which reduce the impacts of grazing invertebrate populations (Boddy 1993).

Invertebrate communities also have the capacity to affect the physiology and functioning of fungal mycelial. Rates of wood decomposition by *P. velutina, P. impudicus* and *H. fasciculare* increased during grazing by *O. asellus, F. candida* and *B. guttulatus,* respectively. This may be the consequence of a fungal compensatory response resulting in increased enzyme activity and nutrient uptake (Hedlund *et al.* 1991; Bengtsson *et al*. 1993) to counteract the impacts of grazers. Increased wood decay in response to grazing has been recorded previously (Chapter 3) and provides further evidence of the stimulatory effects of saprophagous invertebrates on decomposition via their impacts on soil microbes (Mikola *et al*. 2002; Hattenschwiler *et al.* 2005). Impacts of grazing on fungal metabolic activity are, however, not always stimulatory. The ability of woodlice to prevent *R. bicolor* growth altogether in this study will have limited the fungus's ability to produce digestive enzymes

and led to reduced decay rates of wood inocula. This outcome contrasted with the increased decay rates by established *R. bicolor* systems observed during woodlouse grazing in Chapter 3 and highlights that the timing of interactions can not only dictate mycelial foraging consequences, but also fungal activity and nutrient cycling.

4.6 Conclusions

Invertebrate populations, fungal species and timing of interaction can all affect the outcomes of fungus-invertebrate interactions with implications for nutrient mineralisation and cycling (Boddy 1999; Allison & Vitousek 2005; Rotheray *et al.* 2011). The vulnerability of emerging mycelia to specific invertebrate grazers suggests that this may be a rate-limiting step in the development of non-unit-restricted basidiomycetes. The potential of a fungus to forage for, and decompose, organic resources depends on its ability to avoid or withstand grazing interference during mycelial emergence. The varying susceptibilities of different fungi to grazing may enable invertebrates to exert selective pressures and dictate fungal community compositions in soil. The contrasting effects of different grazer populations adds further to the complexity of these decomposer interactions, and suggests that invertebrate species composition will also play a role in determining microbial community functioning. Predicted changes to invertebrate community compositions, under current climate change projections (Jones *et al.* 1998; Hoeksema *et al.* 2000; Wolters *et al.* 2000), are, therefore, likely to have dramatic implications for fungal growth, community structure and decomposition. Comparing the impacts of fungus-grazer interactions on fungal community compositions, and extracellular enzyme activities may be the next step to understanding their implications for functioning and regulation of forest ecosystems.

5. Invertebrate grazing determines enzyme production by basidiomycete fungi

5.1 Abstract

Extracellular enzymes produced by heterotrophic microorganisms in the soil are responsible for the decomposition of organic compounds. Basidiomycete fungi are the primary decomposer agents in temperate wooded ecosystems and contribute extensively to extracellular enzyme activity and nutrient mineralisation within soils. Growth and development of basidiomycete mycelia is influenced by soil-dwelling invertebrate grazers with potential implications for fungal activity and ecosystem functioning. The impacts of four invertebrate species belonging to Isopoda, Myriapoda, Collembola and Nematoda on the production of eight hydrolytic enzymes by four saprotrophic basidiomycetes (*Phanerochaete velutina, Resinicium bicolor* and two strains of *Hypholoma fasciculare*) were compared in a factorial microcosm study. Grazing generally increased enzyme production but invertebrates had species-specific impacts on enzyme activity. The magnitude of grazing influenced enzyme activity; macrofauna (woodlice and millipedes) induced the greatest responses. Enzymatic responses varied markedly between fungi. Grazing enhanced enzyme activity in the exploitative mycelial networks of *P. velutina* and *H. fasciculare*, while the opposite effects were observed in the explorative *R. bicolor* networks. The impacts of soil fauna on nutrient mineralisation depend on fungal community composition. β-Glucosidase, cellobiohydrolase, N-acetylglucosaminidase, acid phosphatase and phosphodiesterase activities were affected most frequently by grazing and invertebrate activity and thus had direct consequences for C, N and P cycling. The results indicate that invertebrate diversity and community composition may influence the spatial distribution and activity of extracellular enzymes with direct implications for nutrient mineralisation and turnover in woodland soils.

5.2 Introduction

Saprotrophic basidiomycete fungi are ubiquitous throughout forest soils. They are the most potent decomposers of cellulose - the main polymeric component of plant cell walls and the most abundant polysaccharide on Earth (Hättenschwiler *et al.* 2005; Baldrian 2008; Baldrian & Valášková 2008). Many non-unit restricted basidiomycetes (i.e. those that can grow out of organic resources in search of new ones) produce large, dynamic mycelial networks, which grow at the soil-litter interface and interconnect discrete wood and litter resources (Boddy 1993). Enzyme production occurs throughout these mycelial networks, and varies both between and within individual systems (Baldrian 2004; Šnajdr *et al*. 2011). Basidiomycete fungi, therefore, influence the activity and spatial distribution of hydrolytic enzymes throughout forest soils.

Extracellular enzymes are responsible for the breakdown of plant cell walls and the mineralisation of complex compounds into simple molecules (e.g. sugars, amino acids or PO_4^{-3}) which can be assimilated (Allison & Vitousek 2005; Caldwell 2005). Enzymes are, therefore, not only vital to the uptake of nutrients by microbes and plants, but also catalyse the initial step in the cycling of carbon and nutrients (Sinsabaugh 1994). Extracellular enzyme activity (EEA) is used as an indicator of soil quality; high activity being associated with regions of high nutrient turnover and primary productivity (Bandick & Dick 1999). Among the most important and widely assayed enzymes are those involved in the decomposition of cellulose into glucose subunits or in the acquisition of nitrogen and phosphorus from organic compounds (Sinsabaugh *et al.* 2008; Caldwell 2005; Toor *et al.* 2003). Because they are linked to C, N, P and S cycling, microbial activity and primary productivity, the factors affecting EEA have been studied extensively due to their implications for forest and agricultural land management (Bandick & Dick 1999; Sinsabaugh *et al.* 2008).

Following enzyme production, efficient uptake of nutrients by basidiomycete fungi can limit the supply of inorganic nutrients to plants with direct consequences for primary productivity (Bardgett 2005). Encounters between basidiomycete mycelia and antagonistic soil organisms can, however, result in the release of these nutrients from otherwise conservative mycelia. Competitive interactions between fungi often lead to lysis of hyphal cell walls and leakage of nutrients (Wells & Boddy 2002), while mycophagous soil fauna ingest mycelia and release

nutrients into the soil in forms that are biologically available (Clarholm 1985). These direct effects of decomposer interactions increase soil nutrient availability but the potential indirect effects on fungal growth and EEA may have greater implications (Boddy 1993). Ecophysiology of foraging basidiomycete mycelial systems is affected by competing microorganisms (Boddy 2000; Baldrian 2004; Hiscox *et al*. 2010) but information regarding the impacts of grazing invertebrates on extracellular enzyme production is limited (Boddy & Jones 2008). Nematodes (*Panagrellus redivivus*) and collembola (*Onychiurus armatus*) influence protease production by *Phanerochaete velutina* and *Mortierella isobellina,* respectively, when growing on agar (Hedlund *et al.* 1991; Dyer *et al.* 1992), but the impacts of these, and other soil fauna, on extracellular enzyme activities in soil are unknown. Numerous studies have highlighted the potential of grazing fauna to increase lignocellulytic activity (Scheu, 1993; Osono, 2007) and nutrient mineralisation (Bardgett & Chan 1999; Mikola *et al*. 2002; Hattenschweiler *et al.* 2005) by litter fragmentation within soil but, to date, none have attempted to quantify or compare the direct impacts of grazing on enzyme production by saprotrophic mycelial networks.

Extensive research on the grazing of mycelial systems in soil by collembola, and to a lesser extent other invertebrates has revealed species-specific, almost idiosyncratic, effects on mycelial growth and activity (Tordoff *et al.* 2006; Chapter 3). In some cases, mycelial extension rates increase in response to grazing by micro-, meso- and macrofauna; this has been attributed to a compensatory response (Hedlund *et al.* 1991; Harold *et al.* 2005). Fungus-mediated wood decomposition rates can also increase during grazing where increased nutrient uptake by fungi enables them to counteract the negative impacts of grazing (Tordoff *et al.* 2006; Chapter 3). Changes in fungal productivity (metabolism, respiration and growth) are likely to be mediated by altered extracellular enzyme production (Hedlund *et al.* 1991) with implications for soil nutrient mineralisation and availability.

In the present study, extracellular enzyme production by fungal mycelia experiencing invertebrate grazing was investigated in laboratory soil microcosms. The basidiomycetes *Phanerochaete velutina* and *Resinicium bicolor,* and two strains of *Hypholoma fasciculare* were used. The fungi were confronted with invertebrates representing the Isopoda, Myriapoda, Collembola and Nematoda. These species all represent common temperate woodland biota. Activities of eight enzymes, associated with hydrolysis of the labile components of soil organic matter, were measured in soil colonised by basidiomycete mycelia

in grazed or ungrazed regimes. The size and hyphal coverage of mycelial networks were also determined. It was predicted that: (i) grazing invertebrates would affect extracellular enzyme production by some basidiomycete systems; and (ii) grazers would have species-specific impacts on fungal enzyme production.

5.3 Materials and methods

5.3.1 Fungal culturing and inoculation

Phanerochaete velutina (Strain P29), *Resinicium bicolor* (Strain Rb1) and *Hypholoma fasciculare* (Strains DD2 and JH) (Cardiff University Culture Collection) were maintained in 13 cm vented Petri dishes on 2% malt extract agar (MEA; 20 g^{-1} Munton and Fiston malt, 15 g^{-1} Lab M agar no. 2). Freshly felled beech wood (*Fagus sylvatica*) was cut into blocks (2 x 2 x 1 cm). These were sterilised (autoclaving at 121°C for three periods of 20 min) and added to fungal cultures. Petri dishes were sealed with Nescofilm® and incubated in the dark for three months at 21°C to allow fungal colonisation.

5.3.2 Invertebrate culturing

Collembola, *Folsomia candida* Willem 1902 (Collembola, Isotomidae) (Cardiff University Culture), millipede, *Blaniulus guttulatus* (Fabricius 1798) (Myriapoda, Julida, Blaniulidae), and woodlouse, *Oniscus asellus* Linnaeus 1758 (Isopoda, Oniscidae) (both collected from Coopers Field, Bute Park, Cardiff (see Chapter 3 for location details)) were kept in 0.8 l containers on a medium of 95% plaster of Paris (Minerva Dental, Cardiff, UK) and 5% activated charcoal (Sigma, Poole, UK). Containers were stored in a dark cupboard at 20°C. Cultures were moistened weekly using deionised water and fed on dried baker's yeast (Spice of Life, Cardiff, UK).

Nematode, *Panagrellus redivivus* (Linnaeus 1767) (Rhabditida, Panagrolaimidae) cultures (UK Parasitology Group, Aberystwyth University) and maintained in 500 ml jars on a medium of porridge (45 g porridge oats moistened with distilled water 75 ml and autoclaved (121°C for 20 min)). Clean nematode suspensions were obtained using wet funnel extraction (Southwood & Henderson 2000). Worms were washed in a solution of 5 ppm benomyl and 30 ppm chlorotetracycline to reduce microbial contamination, and then rinsed in sterile deionised water prior to introduction into the experimental microcosms.

5.3.3 Preparation and inoculation of soil microcosms

Soil, collected to a depth of 20 cm from deciduous woodland (Coed Beddick Enclosure, Tintern, UK (see Chapter 3 for location details)), was sieved on site through a 10 mm mesh and air-dried in plastic trays. Dried soil was sieved through 2 mm mesh and frozen for 24 h at -18°C to kill any remaining soil fauna. Prior to use, soil was re-wetted with 340 ml DH_2O kg $soil^{-1}$. 200 g wet soil was then smoothed and compacted to a depth of 5 mm within 24 x 24 cm bio-assay dishes. Preparation of 125 trays allowed for: (i) five replicates of each interaction (interaction trays); (ii) five control trays of each fungus growing without grazers (fungus controls); (iii) five controls of each invertebrate species with no fungus (invertebrate controls); and (iv) five control trays with no organisms added (soil controls).

Fungus-colonised wood blocks were cleaned of surface mycelia and placed into the centre of soil trays. Once mycelia in the trays for each fungal strain had reached approximately 8 cm diameter any visible fungal contamination was removed, and invertebrates were introduced around the basidiomycete mycelium onto un-colonised soil. As grazers were restricted to 2-D environments, rather than 3-D soil environments, invertebrate numbers added to the microcosms were at the lower end of their respective field densities (Banerjee, 1970; Peterson & Luxton, 1982; Topp *et al.,* 2006; Table 3.1). *Panagrellus redivivus*, *F. candida*, *O. asellus* and *B. guttulatus* were added to microcosms at densities of 16.6 x 10^3, 783, 83 and 83 m^{-2}, respectively. After invertebrate addition, trays were sealed in polythene bags to reduce water loss and microbial contamination. Bags were incubated at 21˚C and 70 % relative humidity.

5.3.4 Soil samples for enzyme analysis

Soil samples were extracted 10 d after invertebrate addition (when substantial grazing had been observed in all trays). Trays were devided into a grid of squares (1 cm^3) with each square representing one sample. 30 samples were taken from each interaction and fungus control tray: of the samples, 10 contained soil colonised by old mycelium (2 cm from the wood block) (OM), 10 had soil colonised by young mycelium (1 cm from hyphal tips) (YM) and 10 were from uncolonised soil (S). Ten samples each of un-colonised soil were also extracted randomly from invertebrate and soil control trays. Samples from the same regions of soil from each tray were mixed and stored in a freezer (-18°C).

5.3.5 Enzyme assays

Extracellular enzyme activity was determined in multiwell plates using a modified method of Vepsäläinen *et al.* (2001). Activities of β-glucosidase, α-glucosidase, cellobiohydrolase, β-xylosidase, N-acetylglucosaminidase, arylsulfatase, acid phosphatase and phosphodiesterase was measured using fluorogenic substrate analogues of 4-methylumbelliferyl (MUF) (Table 5.1). Substrate and standard solutions were prepared fresh before use. Soil samples (0.3g) were homogenised in 50 ml of 0.5 M sodium acetate buffer (pH 5.0) in an ice bath for 3 min at 8000 rev min^{-1} using an UltraTurrax (IKA Labortechnik, Germany). In each of the three technical replicates, 200 µl of soil homogenates were combined with 40 µl of enzyme substrate in dimethylsulfoxide; the final concentration of substrate in the reaction mixture was 500 µM. For the background fluorescence measurement, 200 µl of soil homogenates were combined with 40 µL of MUF standards in dimethylsulfoxide to correct for fluorescence quenching. The plates were incubated at 40°C and fluorescence was recorded at 5 and 125 min after sample addition using a microplate reader (Infinite, TECAN, Austria), with excitation and emission wavelengths of 355 nm and 460 nm, respectively, following Baldrian (2009). This allowed calculation of substrate transformation rate. One unit of enzyme activity was defined as the amount of enzyme catalysing the transformation of 0.1 µM of substrate per minute.

5.3.6 Enzyme descriptions

Cellobiohydrolase (CBH) and 1,4-β-glucosidase (BG) both contribute to the breakdown of cellulose and other β-1,4 glucans (Sinsabaugh *et al.* 2008). CBH cleaves cellobiose dimers from the non-reducing ends of cellulose molecules and BG is responsible for the hydrolysis of cellobiose to form glucose. 1,4-α-Glucosidase (AG) hydrolyses maltose, the product of starch degradation, into glucose monomers. N-acetylglucosaminidase (NAG) is involved in the hydrolysis of chitin and other β-1,4-linked N-acetylglucosamine containing polymers. The role of NAG is analogous to that of BG in cellulose degradation (Sinsabaugh *et al.* 2005). Acid phosphatase (AP) and phosphodiesterase (PDE) are involved in the hydrolysis of organic phosphorus-containing compounds (Toor *et al*. 2003). β-xylosidase (BX) hydrolyzes

Table 5.1: Fluorogenic artificial substrates available commercially (MUF= 4-methylumbelliferone) for the enzyme activity measurements. Corresponding terminal groups are separated from macromolecules and from smaller soluble substrates.

Enzyme	Substrate	Element	Macromolecule degraded
Cellobiosidase (CBH), E.C 3.2.1.91	4-MUF-ß-cellobiopyranoside	Carbon	Cellulose
ß-Glucosidase (BG), E.C. 3.2.1.21	4-MUF-ß-D-glucopyranoside	Carbon	Cellulose
α-Glucosidase (AG), E.C. 3.2.1.20	4-MUF-α-D-glucopyranoside	Carbon	Starch and glycogen
ß-Xylosidase (BX), E.C. 3.2.1.37	4-MUF-ß-D-xylopyranoside	Carbon	Xylane, xylobiose
Chitinase (NAG), E.C. 3.2.1.30	4-MUF-N-acetyl-ß-D-glucosaminide	Carbon and Nitrogen	Breaking ß-1-4-glycosidic bonds in N-acetyl-glucosaminide (chitin) and chitobiose
Phosphodiesterase (PDE), E.C. 3.1.4.1	bis-(4-MUF)-phosphate	Phosphorus	Hydrolysis of phosphate diesters
Acid phosphatase (AP), E.C. 3.2.1.30	4-MUF-phosphate	Phosphorus	Hydrolysis of phosphate diesters
Arylsulphatase (AS), E.C. 3.1.6.1	4-MUF-sulphate	Sulphur	Mineralization of organic sulphur

xylobiose and short chain xylo-oligosaccharides from the non-reducing end to xylose (Saha 2003), thus catalyzing the rate-limiting step in the hydrolysis of xylan (Poutanen & Puls 1988). Arylsulfatase (AS) catalyses the hydrolysis of organic sulfate esters, and plays an important role in the mineralisation and acquisition of organic sulphur (Li & Sarah 2003).

5.3.7 Image capture and analysis

Soil trays were photographed using a Nikon Coolpix 57000 camera, mounted on a stand at a height of 40.5 cm. Images of each tray were captured on the first and final day of each interaction. Images were analysed using IMAGEJ (National Institute of Health, USA). A 2 cm line was drawn against a 1 x 1 cm grid for calibration. Wood blocks, tray edges and surroundings were removed by windowing and the resulting image converted into 8-bit, and then to binary with a manually set threshold. Mycelia and soil were indicated by red and black pixels, respectively, allowing hyphal coverage (cm^2) to be calculated (number of red pixels). Standardization of enzyme activities to hyphal coverage controlled for the varying amounts of mycelium in different soil trays.

5.3.8 Statistical analysis

Activity of each enzyme under young and old mycelia was compared between treatments using Two-Way Analysis of Variance (ANOVA) - conducted in R version 2.10.1 (R Development Core Team 2008) – with fungus and grazer as factors. AP and PDE were not normally distributed (Anderson-Darling Test) and violated assumptions of ANOVA so were log and square root transformed, respectively. Tukeys post-hoc tests were then used to compare the effects of different grazers within individual fungus treatments. Enzyme activities in invertebrate and soil control trays were compared using One-Way ANOVA, as were the effects of grazers on hyphal coverage for each fungal strain. These data were all normally distributed (Anderson-Darling Test) and variances equal (Levene's Test). A Gaussian General Linear Model (GLM) was also performed to test for associations between enzyme production and hyphal coverage in different grazing treatments. This allowed investigation of whether any change in enzyme activity may be a product of changes in fungal biomass during grazing.

5.4 Results

5.4.1 Grazing effects on mycelial distribution

Mycelial distribution varied between fungi. Both *H. fasciculare* DD3 and *P. velutina* produced densely aggregated mycelia, covering the majority of soil within the diameter of their respective networks (Fig. 5.1). In contrast, *R. bicolor* produced thick, loosely aggregated mycelia, displaying a more explorative foraging strategy (Boddy 1999). *Hypholoma fasciculare* JH morphology was an intermediate between these two foraging strategies, producing thick mycelia with branched tips (Fig. 5.1).

Fungi also varied in their susceptibility to grazers. Both *R. bicolor* and *P. velutina* were grazed heavily by *O. asellus* and *F. candida. O. asellus* grazing significantly reduced hyphal coverage of *P. velutina* (One-way ANOVA; $F_{4,20} = 4.978$, $P < 0.001$) and *R. bicolor* ($F_{4,20} = 22.69$, $P < 0.001$). *F. candida* also reduced *P. velutina* ($F_{4,20} = 4.978$, $P = 0.028$) and *R. bicolor* ($F_{4,20} = 22.69$, $P < 0.001$) but to a lesser extent than *O. asellus* (Fig 5.2). The fungi, however, responded very differently to grazing. *P. velutina* displayed a morphological response characterised by increased hyphal branching around mycelial cords (*c.f.* Tordoff *et al.* 2006) while *R. bicolor* showed no responses to the removal or damage of mycelia (Fig. 5.1).

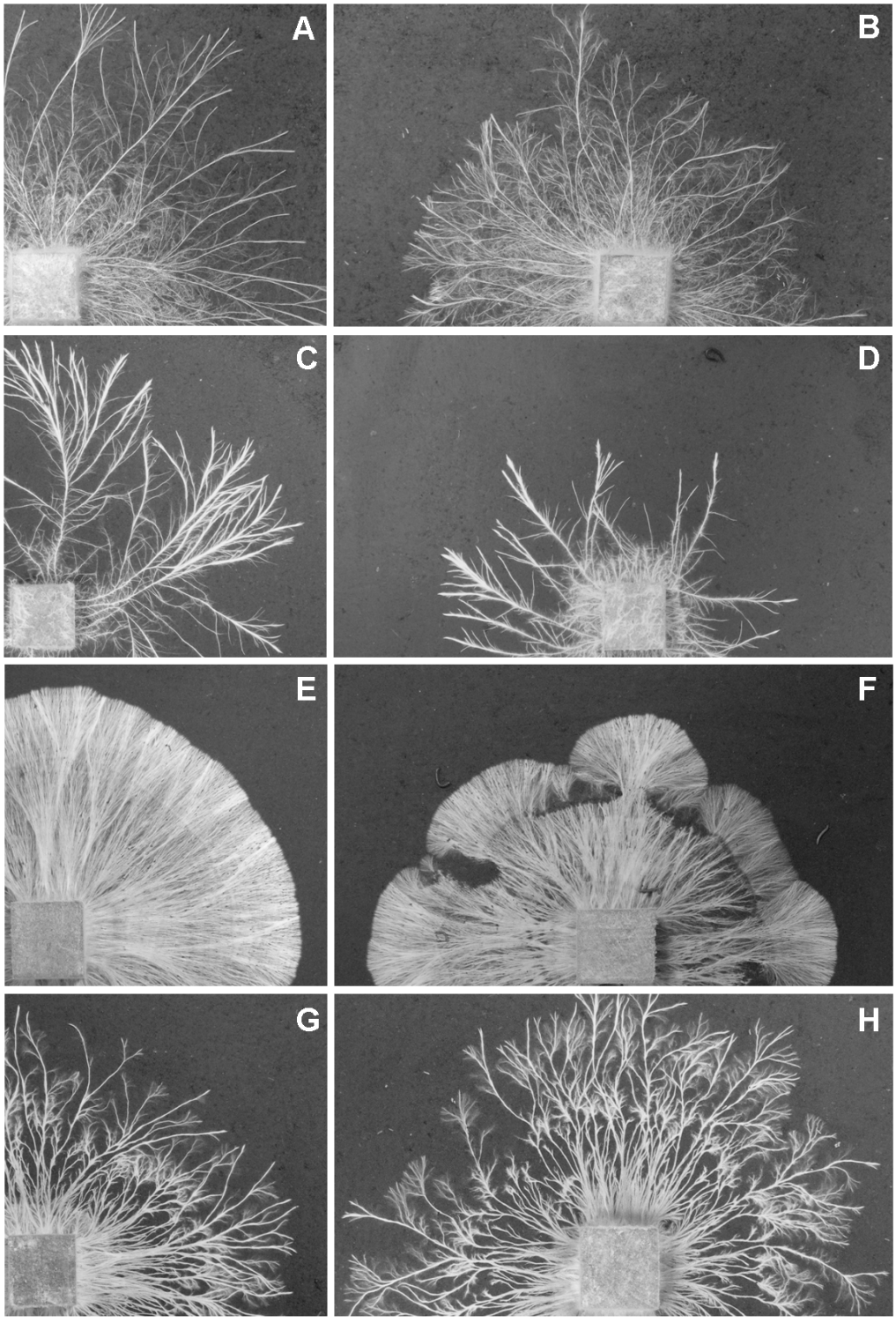

Fig. 5.1: Digital images showing growth of *Phanerochaete velutina* (A, B), *Resinicium bicolor* (C, D), *Hypholoma fasciculare* DD3 (E, F) and *Hypholoma fasciculare* JH (GH) in ungrazed (A, C, E, G) and grazed (B, D, F, H) treatments after 10 d of growth from 2 x 2 x 1 cm wood blocks on 24 x 24 cm soil trays.

B. guttulatus grazing caused the greatest damage to the two strains of *H. fasciculare* (DD3: $F_{4,20} = 36.86$, $P < 0.001$; JH: $F_{4,20} = 4.308$, $P = 0.005$), although *O. asellus* also reduced hyphal coverage of *H. fasciculare* JH ($F_{4,20} = 4.308$, $P = 0.044$). Both strains responded to grazing in a similar manner to *P. velutina,* increasing production of fine hyphae and outgrowth from grazed regions. Responses of *H. fasciculare* DD3 were, however, more pronounced than *H. fasciculare* JH (Fig. 5.1).

5.4.2 Enzyme activities in control trays

Enzyme activities in un-colonised soil (invertebrate and soil control) trays were highly variable. There were no significant differences ($P > 0.05$) in the activity of any enzyme between invertebrate and soil control trays. No invertebrate species directly affected enzyme activity in un-colonised soil.

In fungus controls, BG activities were significantly ($P \leq 0.05$) greater in soil colonised by all fungi than in un-colonised soil. NAG, CBH, BX, AP and PDE activities increased under old mycelia of certain fungi (Table 5.2a), while AS and AS activities were not significantly ($P > 0.05$) different in colonised and un-colonised soil. BG, NAG and AP activities increased under young mycelia of specific fungal strains while AG, CBH, BX, PDE and AS were not affected (Table 5.2b).

5.4.3 Enzyme activities in interaction trays

Enzyme activities in colonised soil were affected by all invertebrate species (Table 5.2). These were not significantly (GLM; $P > 0.05$) influenced by hyphal coverage in any fungal treatment. Recorded changes in enzyme production by fungi during grazing were, therefore, not due to changes in fungal biomass.

Enzymatic responses to grazing varied between fungi. All recorded changes in soil colonised by *P. velutina* and *H. fasciculare* DD3 represented increases in enzyme activity. Effects ranged from being insignificant (AS) to being almost twofold (BG, AP) greater than in ungrazed fungus controls (Fig 5.3). In contrast, enzyme activity under *R. bicolor* cords was always reduced by invertebrate grazing. Enzyme production by *H. fasciculare* JH was generally unaffected by grazing; only AP activity increased during millipede grazing (Table 5.2).

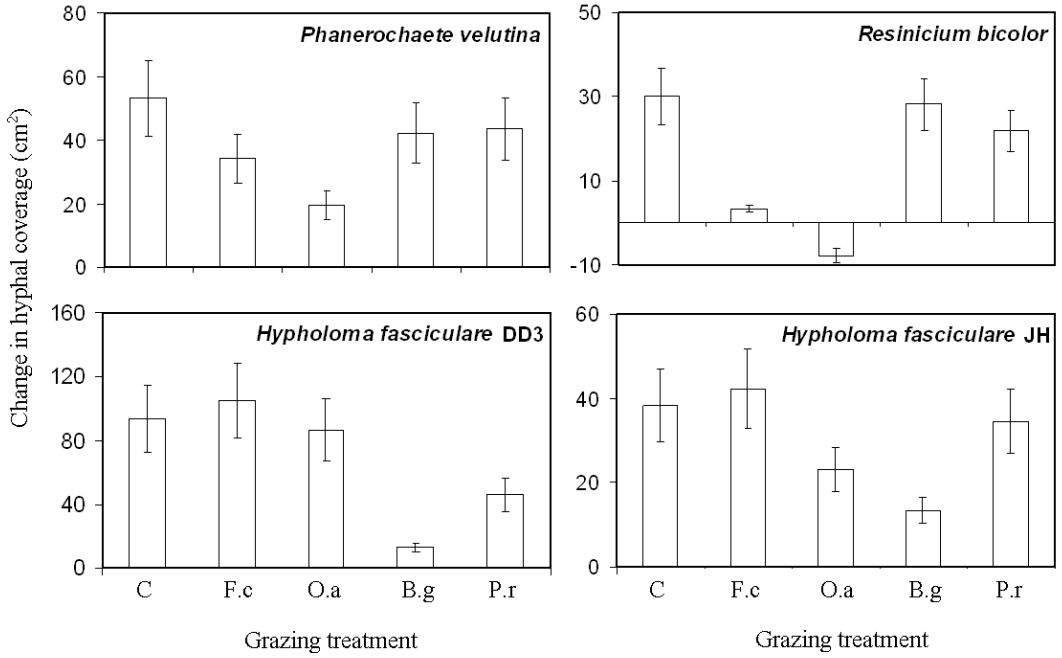

Fig. 5.2: Mean (± SE) change in hyphal coverage between Day 1 (when invertebrates were added) and Day 10 (when soil samples were taken) of *Phanerochaete velutina, Resinicium bicolor, Hypholoma fasciculare* DD3 and *H. fasciculare* JH grazed by *Folsomia candida* (F.c), *Oniscus asellus* (O.a), *Blaniulus guttulatus* (B.g), *Panagrellus redivivus* (P.r), fungus-only control (C).

For each fungal strain, the invertebrate species which were responsible for the greatest reduction in hyphal coverage also exerted the greatest influences on enzyme activity. *Oniscus asellus* caused the greatest number of significant ($P \leq 0.05$) effects to *P. velutina* and *R. bicolor*, significantly ($P \leq 0.05$) affecting the activities of five and six enzymes, respectively (Fig 5.3). *Folsomia candida* significantly ($P \leq 0.05$) increased BG, CBH and AP activities in soil colonised by *P. velutina* and AP activity under *H. fasciculare* DD3 mycelia (Table 5.2). *Blaniulus guttulatus* exerted the greatest number of changes to enzyme activity under both *H. fasciculare* strains. Millipedes stimulated the production of BG, BX and CBH by *H. fasciculare* DD3 mycelia and were the only species to influence the activity of any enzyme in *H. fasciculare* JH trays (Fig 5.3).

Most enzymatic responses to grazing were similar in young and old regions of mycelia (Fig 5.3). BG, AP and PDE activities, for example, increased under young and old *P. velutina*

Table 5.2: Effects of invertebrate grazers on enzyme production by saprotrophic basidiomycetes growing in soil microcosms. P-values of statistically significant (ANOVA; $P \leq 0.05$) effects of grazers on production of eight enzymes (BG: 1,4-β-glucosidase; AG: 1,4-α-glucosidase; CBH: Cellobiohydrolase; BX: 1,4-β-xylosidase; NAG: N-acetylglucosaminidase; AS: Arylsulfatase; AP: Acid phosphatase; PDE: Phosphodiesterase) by old (a) and young (b) mycelia of four fungi (Pv: *Phanerochaete velutina*; Rb: *Resinicium bicolor*; Hf DD3: *Hypholoma fasciculare* DD3; *H. fasciculare* JH). Enzyme activities in grazing (Pr: *Panagrellus redivivus*; Fc: *Folsomia candida*; Oa: *Oniscus asellus*; Bg: *Blaniulus guttulatus*; and So: un-colonised soil) treatments were compared against those in ungrazed fungus control treatments. Arrows indicate the direction of response and dashes indicate no significant differences from fungus-only controls.

5.2 a	Invert	BG	AG	CBH	BX	NAG	AS	AP	PDE
Pv	**Pr**	-	-	-	-	-	-	-	-
	Fc	0.033↑	-	0.043↑	-	-	-	0.045↑	-
	Bg	-	-	-	-	-	-	-	-
	Oa	0.001↑	-	0.045↑	-	0.018↑	-	0.003↑	0.038↑
	So	<0.001↓	0.021↓	0.04↓	0.022↓	0.026↓	-	0.033↓	0.041↓
Rb	**Pr**	-	-	-	-	-	-	0.041↓	-
	Fc	-	-	-	-	-	-	-	-
	Bg	-	-	-	-	0.009↓	-	-	-
	Oa	0.027↓	0.044↓	-	0.029↓	0.029↓	-	-	-
	So	<0.001↓	0.048↓	-	-	0.001↓	-	0.032↓	-
Hf DD3	**Pr**	-	-	-	-	-	-	-	-
	Fc	-	-	-	-	-	-	-	-
	Bg	0.041↑	-	<0.032↑	0.044↑	-	-	-	-
	Oa	-	-	-	-	-	-	-	-
	So	0.021↓	-	0.003↓	0.029↓	0.048↓	-	-	-
Hf JH	**Pr**	-	-	-	-	-	-	-	-
	Fc	-	-	-	-	-	-	-	-
	Bg	-	-	-	-	-	-	-	-
	Oa	-	-	-	-	-	-	-	-
	So	0.024↓	-	-	-	-	-	0.004↓	-

5.2 b	Invert	BG	AG	CBH	BX	NAG	AS	AP	PDE
Pv	**Pr**	-	-	-	-	-	-	-	-
	Fc	<0.001↑	-	-	-	-	-	0.021↑	-
	Bg	-	-	-	-	-	-	-	0.031↑
	Oa	0.001↑	-	-	-	-	-	0.033↑	0.045↑
	So	-	-	-	-	-	-	0.049↓	-
Rb	**Pr**	-	-	-	-	-	-	-	-
	Fc	-	-	-	-	-	-	-	-
	Bg	-	-	-	-	0.019↓	-	-	-
	Oa	0.01↓	-	-	-	-	-	0.041↓	0.028↓
	So	0.024↓	-	-	-	0.045↓	-	-	0.042↓
Hf DD3	**Pr**	-	-	-	-	-	-	0.028↑	0.033↑
	Fc	-	-	-	-	-	-	<0.028↑	-
	Bg	0.046↑	-	-	-	-	-	-	-
	Oa	-	-	-	-	-	-	-	-
	So	-	-	-	-	-	-	-	-
Hf JH	**Pr**	-	-	-	-	-	-	-	-
	Fc	-	-	-	-	-	-	-	-
	Bg	-	-	-	-	-	-	0.044↑	-
	Oa	-	-	-	-	-	-	-	-
	So	0.044↓	-	-	-	-	-	0.004↓	-

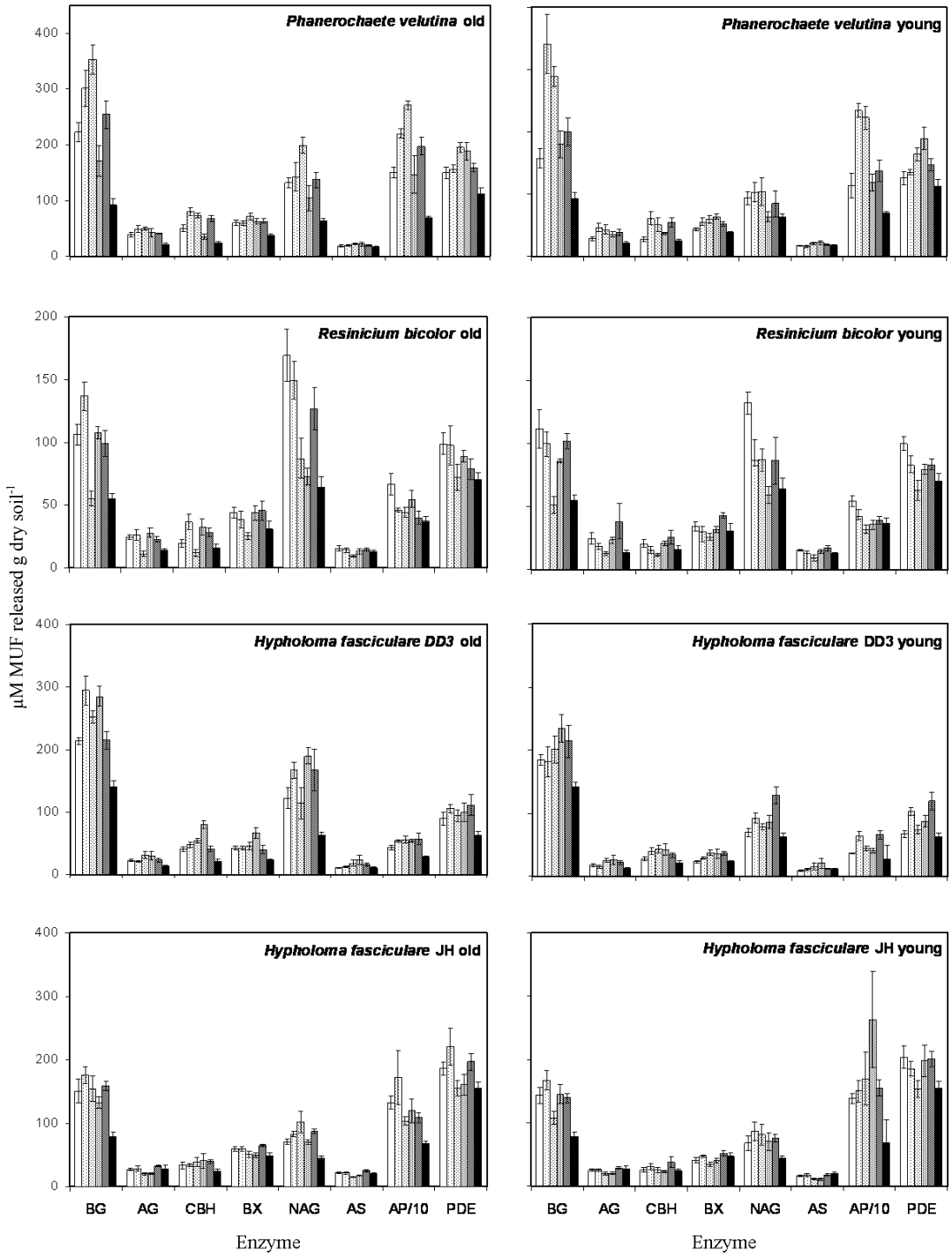

Fig. 5.3: Activities of 1,4-β-glucosidase (BG), 1,4-α-glucosidase(AG), Cellobiohydrolase (CBH), 1,4-β-xylosidase (BX), Arylsulfatase (AS), N-acetylglucosaminidase NAG, Acid phosphatase (AP) and (PDE) Phosphodiesterase (PDE) enzymes in soil colonised by *Phanerochaete velutina, Resinicium bicolor, Hypholoma fasciculare* strains DD3 and JH. Mean and standard errors are shown for each enzyme activity in young and old mycelia during fungus-only control (□), *F. candida* (⊡), *O. asellus* (⊡), *Blaniulus guttulatus* (▦), and *Panagrellus redivivus* (▩) grazing treatments as well as un-colonised soil (■). AP values were divided by 10 to limit empty space.

mycelia during *O. asellus* grazing relative to un-grazed controls. There was, however, one clear deviation from this trend as BX activity was not affected by grazing under young mycelia, but changed in soil colonised by old *R. bicolor* and *H. fasciculare* DD3-mycelia (Fig 5.3).

Activities of all enzymes except AS were significantly ($P \leq 0.05$) affected by some grazing treatments (Fig. 5.3). Certain enzymes were, however, more frequently affected. AP activity was affected by grazing in all four fungi, while BG and PDE activities were significantly ($P \leq 0.05$) affected in soil colonised by *P. velutina, R. bicolor* and *H. fasciculare* DD3 but not *H. fasciculare* JH. NAG and CBH activities were influenced less frequently; both were affected in soil colonised by *P. velutina,* and *R. bicolor* or *H. fasciculare* DD3, respectively.

5.5 Discussion

Enzyme activity in soil colonised by basidiomycete mycelia was affected by grazing. Of the 32 significant grazing effects recorded, 72% represented an increase in specific enzyme activity. Decomposer invertebrates are known to increase decomposition rates by comminution; fragmentation of litter increases the surface area available to primary decomposers, indirectly stimulating microbial activity (Mikola *et al*. 2002; Hättenschwiler *et al.* 2005). The stimulated enzyme production by basidiomycete fungi during invertebrate grazing in the present study represents a more direct mechanism by which soil fauna stimulate organic matter decomposition. Although all grazers influenced mycelial enzyme production, at least to some extent, impacts varied dramatically between invertebrate species. This may be related to grazing intensity. For example, the greatest number of changes to *P. velutina* and *H. fasciculare* DD3 enzyme production were induced by woodlice (*O. asellus*) and millipedes (*B. guttulatus*), respectively - the same invertebrate species that reduced hyphal coverage of these fungi most markedly in this and previous studies (Chapters 3 and 4). The pronounced effects of these larger invertebrates concurs with Bradford *et al.* (2002) which identified macrofauna as a particularly influential group in terms of decomposition and nutrient cycling. The variety of other factors which influence invertebrate grazing potential (such as population dynamics, mouth-part morphology or feeding preference; Kaneko *et al.* 1998) are also reflected in the stochastic nature of enzymatic responses in the present study. Invertebrates, therefore, have species-specific impacts on fungal enzyme production; spatial organisation

and community compositions of soil fauna will have direct consequences for nutrient mineralisation within forest soil.

From a fungal perspective, impacts of grazing were less variable; enzyme activities under *H. fasciculare* DD3 and *P. velutina* mycelia always increased during grazing while activities under *R. bicolor* were always reduced. Mechanistic damage caused by grazing may have either resulted in intracellular enzyme leakage or liberation of cell contents with subsequent increase in microbial enzyme production to digest the substrate (Bucht & Eriksson 1968). Both would lead to increased enzyme production by all damaged hyphae (Wells & Boddy 2002). The striking contrast between changes in enzyme activity under different fungi, however, suggest that enzymatic changes were the result of specific fungal responses to grazing rather than mechanistic damage. This has direct implications for nutrient cycling in different ecosystems; effects of mycophagous soil fauna on fungal-mediated nutrient mineralisation will vary between habitats, depending on the dominant fungal species present. The presence of grazers in soils dominated by *H. fasciculare* or *P. velutina* will, for example, lead to increased enzyme activity, nutrient mineralisation and productivity, with the opposite effects in *R. bicolor*-dominated ecosystems.

The enzymatic response of a fungus may relate to its growth and activity during invertebrate invasion (Hedlund *et al.* 1991). *H. fasciculare* DD3 and *P. velutina* both exhibit an 'exploititive' growth strategy, with dense mycelia employed to maximise the chance of encountering resources over small spatial scales (Boddy 1999). Both species show increased growth (Tordoff *et al.* 2006; Harold *et al.* 2005) and wood decay (Chapter 3) rates during grazing. In contrast, the long-range 'explorative' growth of *R. bicolor* (Boddy 1993) is often stunted by grazing invertebrate populations (Tordoff *et al.,* 2006; A'Bear *et al.,* 2010) and this is coupled with reduced decay rates of wood resources (Chapter 4). In the present study, the exploitative and explorative functional groups exhibited opposite enzymatic responses during grazing. Increased nutrient mineralisation and uptake by exploitative fungi will facilitate compensatory growth responses (Hedlund *et al.* 1991), while the cessation of enzyme production (resulting in the decrease of their activity) in explorative foragers may represent a means of conserving energy (Allison & Vitousek 2005) by species, which are less efficient at attaining nutrients from the soil (Boddy 1993; 1999). This suggests that it may be the diversity of functional groups, rather than fungal species, which dictates the processing of nutrients, especially in fauna-rich regions of soil.

Enzyme responses to grazing varied not only between, but within fungal species. Variation in the responses of different *H. fasciculare* strains to grazing highlights the complexity of these grazing interactions. The potential of an invertebrate community to influence nutrient mineralisation will depend on the inter- and intraspecific fungal diversity within soil. Differential responses of mycelia within the same fungal colony add further to this complexity. Increased enzyme production by old, compared to young mycelia has been recorded in ungrazed systems (Šnajdr *et al*. 2011) but, in the present study, both mycelial regions were equally responsive to grazing. This was not predicted because the majority of invertebrate species selectively graze fresh hyphal tips, rather than thickened mycelial cords (Wiggins *et al.* 1979; Hiol Hiol *et al.* 1994; Tordoff *et al.* 2006). This selective grazing was also observed in the present study, supporting Hedlund *et al.* (1991), who suggested that grazers may influence extracellular enzyme production in un-grazed regions of mycelium. This, and the potential for cord-forming basidiomycete fungi to extend tens of metres across the soil (Boddy, 1999), suggests that localised grazing by soil fauna may influence nutrient mineralisation over large spatial scales, as well as in their immediate vicinity.

With the exception of AS, grazing influenced all assayed enzymes to some extent. Activities of BG, NAG and AP were stimulated more consistently than others; these are probably involved in the reconstruction and re-growth of damaged hyphae. Changes in N-acetylglucosaminidase and acid phosphatase activities, for example, were recorded in all fungal species during grazing. Both enzymes play an important role in the breakdown and re-formation of internal bonds during cell division (Reyes *et al.* 1990; Duo-Chaun 2006) and growth of fungal hyphae (Adams 2004). These hydrolytic enzymes are also associated with wall plasticity (Adams 2004) and are likely to mediate the morphological growth responses recorded previously in *P. velutina* and *H. fasciculare* (Harold *et al.* 2005; Tordoff *et al.* 2006) during grazing. Energy and nutrient requirements of fungi will also change during grazing. Breakdown of hyphae can result in the loss of phosphorus; increased phosphodiesterase and acid phosphatase activities may be a response to the resulting high phosphorous concentrations in the surrounding soil. Similarly, β-glucosidase and cellobiohydrolase activities were stimulated by grazing in *H. fasciculare* DD3 and *P. velutina* microcosms. These will facilitate the increased glucose uptake required during the energetically expensive growth responses employed to counteract grazing (Burns 1982). The stresses associated with grazing will influence the production of specific extracellular enzymes by fungi and indirectly

affect the mineralisation of nutrients within soil. The consistent changes to enzymes involved in cellulose decomposition and phosphate acquisition during grazing suggest that mycophagous fauna may be particularly influential in the cycling of carbon and phosphorus in woodland ecosystems.

5.6 Conclusion

This study points to a mechanism behind the stimulatory effects of soil invertebrates on fungus-mediated decomposition and nutrient turnover (Scheu, 1993; Bardgett & Chan, 1999; Mikola *et al*., 2002). The analyses indicate that the enzymatic potential for hydrolyzing the labile components of soil organic matter is tied to both basidiomycete and invertebrate community composition. Generally, grazing was associated with an increase in enzyme production but individual effects varied between invertebrate species. Increased grazing intensity leads to greater frequency of enzymatic responses, highlighting macrofauna (millipedes and woodlice) as a particularly influential group in terms of wood and litter decomposition (Scheu, 1993; Bradford *et al.,* 2002). These effects were, however, strongly dependent on the fungal species. Contrasting enzymatic responses of different fungal functional groups mean that the impacts of grazing soil fauna vary between fungal communities. These trends suggest that the predicted changes in invertebrate (Wolters *et al.,* 2000) and fungal (Gange *et al.,* 2007) community composition as a result of global climate change will indirectly affect EEA in soil. Retaining high levels of invertebrate species, and functional group diversity is, therefore, not only vital to maintaining fungal biomass and activity, but also nutrient mineralisation, primary productivity and carbon storage in forest ecosystems.

6. Outcomes of fungal interactions are determined by soil invertebrate grazers

6.1 Abstract

Saprotrophic fungal community composition, determined by the outcome of competitive mycelial interactions, is one of the many key factors affecting soil nutrient mineralisation and decomposition rate. Fungal communities are not generally predicted to be regulated by top-down factors such as predation, but rather by bottom-up factors including resource availability. This study shows that invertebrate grazers can exert selective pressures on fungal decomposer communities in soil, reversing the outcomes of competitive interactions. By feeding selectively on the cord-forming fungus *Resinicium bicolor,* isopods prevented the competitive exclusion of *Hypholoma fasciculare* and *Phanerochaete velutina* in soil and wood. Nematode populations also reversed the outcomes of competitive interactions by stimulating growth of less competitive fungi. These represent two opposing mechanisms by which soil fauna may influence fungal community composition and diversity. Factors affecting soil invertebrate communities will have direct consequences for fungal-mediated nutrient cycling in woodland soils.

6.2 Introduction

The key influences of plant diversity and community composition on ecosystem processes are well established (Tilman *et al.* 1997; Hooper & Vitousek 1997) and, as a result, the factors (biotic and abiotic) affecting them have been extensively explored (Clark & Tilman 2008; Yang *et al.* 2011). In contrast, despite the acknowledged contribution of belowground microbial communities to processes including carbon and nutrient cycling (Wardle 2002; Bardgett 2005; van der Heijden *et al.* 2008), the biotic factors influencing their compositions are less well understood (Wardle 2006). Saprotrophic basidiomycete fungi are the primary decomposing agents in temperate woodland ecosystems (Hättenschwiler *et al.* 2005; Baldrian & Valášková 2008). Their filamentous mycelial networks grow throughout the soil-litter interface, forming systems which contribute significantly to total ecosystem biomass and respiration (Post *et al.* 1982; Bardgett 2005). During mycelial extension, competitive interactions take place at a distance, via antagonistic volatile organic compound production, or following mycelial contact, and commonly result in the replacement of one fungus by another (Boddy 2000). The outcomes of these mycelial interactions determine fungal dominance and community composition in litter resources and soil (Boddy 2000). Species-specific fungal enzyme production and respiration rates suggest that factors affecting the outcomes of these competitive interactions are likely to have consequences for decomposition rates, nutrient mineralisation and the flux between terrestrial and atmospheric carbon pools (Hättenschwiler *et al.* 2005; Gessner *et al.* 2010).

The networks of interacting mycelia that pervade woodland soil represent the primary ecosystem nutrient pool for soil-dwelling animals (Pollierer *et al.* 2009). The effects of 'grazers' on fungal community structure are, however, relatively poorly understood. The Nutrient-enrichment Model (Moore *et al.* 2003) argues that, unlike bacteria, fungal communities are not regulated by top-down control (predation), but by bottom-up factors, including resource availability. By virtue of their large biomass and biochemical defences, it is predicted that fungi are relatively resistant to grazing (Wardle & Yeates 1993) and that nutrients are conserved within vast mycelial networks (Boddy 1999). This is supported by empirical studies suggesting that, although mesofauna (collembola and mites) feed selectively on specific fungi, the grazing pressures exerted are not strong enough to alter fungal community composition (Parkinson *et al.* 1979; Whittaker 1981; Kaneko *et al.* 1998). Newell (1984a; 1984b) provided some evidence that collembola may differentially influence the

competitive abilities of interacting fungi. Selective grazing of the dominant fungus (*Marasmius androsaceus*) increased the relative abundance of a less palatable species (*Mycena galopus*) colonising Sitka spruce (*Picea sitchensis*) needles. Collembola have subsequently been found to stimulate the progression rates of mycelial interactions (Klironomos *et al.* 1992), but no study has shown that grazing can lead to the complete replacement of a formerly dominant fungus by a less competitive opponent (McLean *et al.* 1996; Wardle 2006).

More recent studies involving a wider range of invertebrate species suggest that macrofauna (including isopods and millipedes) consistently exert stronger grazing pressures on individual mycelial systems than smaller mesofauna (Chapters 4 and 5). Effects vary dramatically between basidiomycete species; fungal palatability, based on mycelial biochemistry and morphology (Hiol Hiol *et al.* 1994), determines whether foraging systems are consumed entirely or ignored by grazers (Chapter 3). Such high intensity grazing has been predicted to exert selective pressures on interacting fungi and drive changes in community composition (Kaneko *et al.* 1998). Moreover, the stimulatory effects of low intensity microfauna (nematode) grazing on the extension rates of selected fungi, analogous to the compensatory growth responses seen in plants during herbivory (McNaughton 1983), highlight the need to investigate the effects of a broader range of invertebrate taxa on fungal community composition and functioning.

In the present study, the potential of invertebrate taxa representing the Isopoda, Myriapoda, Collembola and Nematoda to affect the outcomes of inter- and intraspecific fungal interactions in soil and wood were explored. All fungi used are common within temperate woodland ecosystems (Boddy 1999, Boddy 2000) and were isolated originally from UK forest soil. A fully-factorial microcosm experiment was used to test three hypotheses: (i) selective grazing can reverse the outcomes of competitive fungal interactions with subsequent shifts in fungal species composition; (ii) as a result of increased grazing intensity, macro-fauna will exert greater selective pressures on fungal communities than meso- and microfauna; and (iii) grazing will influence fungal-mediated wood decay.

6.3 Materials and methods

6.3.1 Experimental design

To test Hypotheses (i) and (ii), four cord-forming basidiomycete fungi were allowed to grow from wood blocks and interact (in pair-wise combinations) within 2-D soil microcosms. 150 microcosms provided a balanced, factorial design, with all six fungal combinations subject to five grazing treatments (collembola, nematode, isopod, millipede and un-grazed control), each replicated five times (i.e. 6 interactions x 5 treatments x 5 replicates). Wood decay rates were determined at the end of the experiment to test Hypothesis (iii).

6.3.2 Fungal culturing and inoculum preparation

Fungal isolates, *Hypholoma fasciculare* (Strains DD3 and JH) (Huds.: Fr.), *Phanerochaete velutina* (DC.: Pers.) and *Resinicium bicolor* (Abertini and Schwein.: Fr.) (Cardiff University Culture Collection), were subcultured in non-vented 9 cm diameter Petri dishes on 2% malt extract agar (MEA; 15 g l^{-1} Lab M agar no. 2, 20 g l^{-1} Munton and Fiston malt). Freshly-cut beech (*Fagus sylvatica*) wood blocks (2 x 2 x 1 cm) were stored at -18 °C, and autoclaved at 121°C for 20 minutes prior to use. Sterilized wood blocks were then added to fungal cultures. Petri dishes were sealed with Nescofilm®, and incubated in the dark at a constant temperature of 20 °C for 3 months prior to experimental use.

6.3.3 Invertebrate collection and culturing

Folsomia candida Willem 1902 (Collembola) (Cardiff University Culture) were cultured in 0.8 l containers on a medium of 95 % plaster of Paris (Minerva Dental, Cardiff, UK) and 5% activated charcoal (Sigma, Poole, UK). Cultures were fed weekly on dried baker's yeast. *Blaniulus guttulatus* (Fabricius 1798) (Myriapoda) and *Oniscus asellus* Linnaeus 1758 (Isopoda) (collected from Coopers Field, Bute Park, Cardiff, UK (see Chapter 3 for location details)) were kept in 2 l plastic pots containing compost. All containers were stored in the dark at 20°C and moistened weekly using deionised water (DH_2O). Before introduction into experimental microcosms all three species were starved for 24 h in pots with fresh plaster of Paris.

Panagrellus redivivus (Linnaeus 1767) (Nematoda) cultures (supplied by UK Parasitology Group, Aberystwyth University) were maintained in 500 ml jars on a medium of porridge oats (45 g) and DH_2O (75 ml) which had been autoclaved (121°C for 20 min) prior to nematode

addition. Before introduction into experimental microcosms nematode suspensions were extracted using wet funnel extraction (Southwood & Henderson 2000). Worms were then washed in a solution of 30 ppm chlorotetracycline and 5 ppm benomyl to reduce bacterial and fungal contamination associated with the culture medium (Dyer *et al.* 1992). A final funnel extraction was performed to acquire suspensions of free-living *P. redivivus* (1000 ml^{-1}).

As well as being good model species, the invertebrates used in this study are common representatives of their respective taxa in temperate European soils (Dyer *et al.* 1992; Jones & Hopkin 1990; Bradford *et al.* 2002; Fountain & Hopkin 2005). All have been shown to influence the growth and physiology of saprotrophic basidiomycete fungi in soil (Chapter 3).

6.3.4 Microcosm preparation, inoculation and running

Loamy soil was collected from deciduous woodland (Coed Beddick Enclosure, Tintern, UK (see Chapter 3 for location details)) to a depth of 20 cm and sieved on site through a 10 mm mesh. This was air-dried in plastic trays and sieved again through 2 mm mesh before being frozen over night at -20°C to kill any remaining fauna. Prior to use, soil was re-wetted with DH_2O (340 ml kg $soil^{-1}$) giving a final water potential of -0.012 MPa. 200 g moistened soil was then compacted and smoothed to a depth of 5 mm into 24 x 24 cm bio-assay dishes.

Fungus-colonised wood blocks were removed from agar cultures. Densities (dry weight/fresh volume; g cm^{-3}) of seven blocks colonised by each fungus were determined at 0 d. The remaining blocks were cleaned of surface mycelia before being added to soil trays; they were placed 9 cm from opposing corners on a diagonal line ensuring a gap of 8 cm between wood blocks of interacting fungi. Wood block addition dates varied depending on the species-specific mycelial extension rates of the fungi (Tordoff *et al.* 2008). This was done to ensure that emerging mycelia met after 4 cm growth. Once opposing mycelia in 50% of the trays for each fungal interaction had met for 2 d, invertebrates were introduced onto un-colonised regions of soil. As grazers were restricted to a 2-D environment, population numbers added to microcosms represented low estimates of field densities of each taxon (Table 3.1). *Panagrellus redivivus*, *F. candida*, *B. guttulatus* and *O. asellus* were added at densities of 16.6 x 10^3, 783, 83 and 83 m^{-2}, respectively. Trays were then stacked in polythene bags to reduce water loss and stored at 20˚C and 70 % relative humidity. Microcosms were re-wetted weekly to a constant weight with DH_2O. The experiment was concluded after 63 d, and each wood block was then cut in half; one half was used for re-isolation of colonising fungi and the

second half used to determine wood block density enabling estimation of wood decay rate (g cm^{-3} d^{-1}).

6.3.5 Image capture and analysis

Digital images were captured after 0, 3, 7, 14, 21, 35, 49 and 63 d using a Nikon Coolpix 57000 camera, mounted on a stand at a height of 39.5 cm. These were subsequently analysed using IMAGEJ (National Institute of Health, USA). A 5 cm line was drawn against a ruler for calibration. The extents of mycelia growing through a 90° angle were estimated using the mean length of four lines (30° apart) drawn from the centre of each wood block to hyphal tips. Extension rates (cm d^{-1}) were determined for each interaction until mycelia from any replicate reached the opposing wood block.

6.3.6 Determination of interaction outcomes

Interaction outcomes on the soil were determined by observing which mycelia had reached the opposing wood block after 63 d. Outcomes were classified as: (i) replacement, where mycelia of one fungus was killed and replaced by its opponent; (ii) overgrowth, where one fungus had grown over another and caused the cessation of growth without killing its opponent; (iii) mutual replacement, where mycelia from both opponents had grown into the opponent's territory; and (iv) deadlock, where neither fungus had gained territory of the other (Fig. 6.1). Overgrowth and replacement were recorded as being a "win" if the aggressor reached the opposing woodblock. Mutual replacement and deadlock were recorded as a "draw" as both, or neither, wood block had been reached.

Wood chips taken from the freshly-cut surfaces of halved wood blocks were placed onto 9 cm Petri dishes containing 2 % MEA. These were incubated in the dark for 7 d at 20ºC. Emerging mycelia were identified by visual inspection. A "win" was recorded if both wood blocks from a single microcosm had been colonised by one fungus (i.e. if one fungus had replaced its opponent and successfully defended its own resource). If the two wood blocks were colonised by different fungi (i.e. neither or both fungi had been replaced) the interaction was recorded as a "draw".

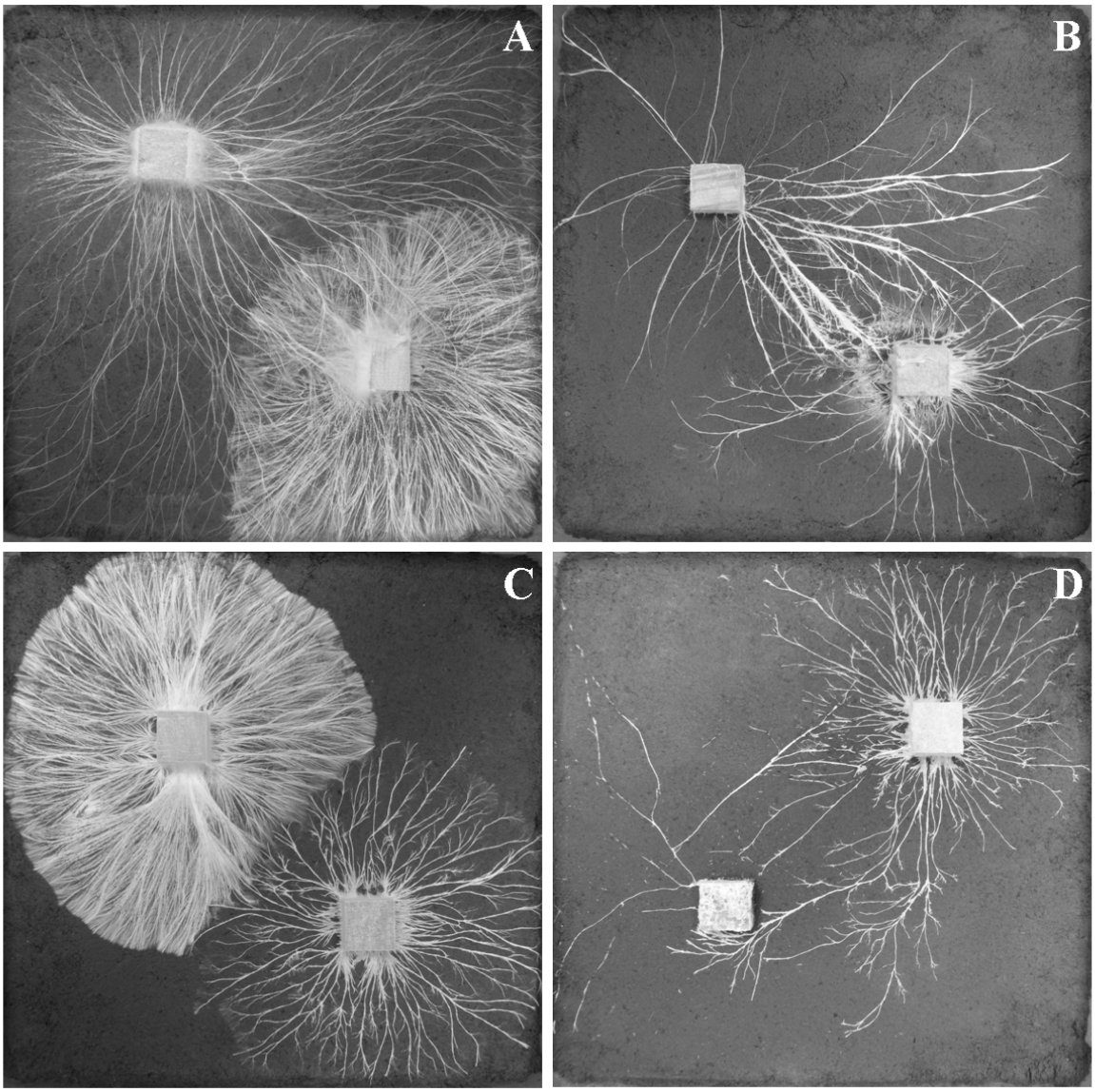

Fig. 6.1: Digital images showing examples of the four possible outcomes of mycelial interactions in soil: overgrowth (A) of *Hypholoma fasciculare* DD3 (right) by *Phanerochaete velutina* (left), replacement (B) of *H. fasciculare* JH (right) by *Resinicium bicolor* (left), deadlock (C) between *H. fasciculare* JH (right) and DD3 (left), and mutual replacement (D) between *H. fasciculare* JH (right) and *Resinicium bicolor* (left).

6.3.7 Feeding study

A further 126 soil trays were prepared (as above) to observe the distribution of *F. candida, B. guttulatus* and *O. asellus* in relation to the position of two opposing fungal resources. Seven

replicates were established of each of six fungal combinations (*R. bicolor, P. velutina* or *H. fasciculare* DD3 paired against itself and against a different species in a fully factorial design) for each invertebrate species. Once mycelia had extended to 2 cm from the centre of each wood block, 2 isopods, 2 millipedes and 10 collembola were introduced into separate microcosms. These were then maintained in the dark at 20°C and 70 % relative humidity. Over the following 12 h, the number of invertebrates grazing within a 4 cm diameter circle around each woodblock was recorded every hour.

6.3.8 Statistical analysis

Multinomial Logistic Regression (Minitab 15) was used to compare frequencies of three possible interaction outcomes (win for fungus 'a', win for fungus 'b' or draw) in different grazer treatments in soil and wood. When only two outcomes were recorded for any given comparison, Binary Logistic Regression was used.

The general relationship between extension rates of competing fungi in un-grazed interactions was investigated using Pearson's Correlation (Minitab 15). Mycelial extension rates of both fungi in each mycelial interaction were then compared across invertebrate treatments using Analysis of Covariance (ANCOVA; General Linear Model; Minitab 15) with time (days after invertebrate addition) as a covariate; data not meeting assumptions of linearity were log-transformed. Chi squared ($\chi 2$) tests were used to compare the numbers of invertebrates recorded grazing on each fungus during the feeding study.

The general relationship between fungal wood decay and extension rate was investigated using Pearson's correlation. Wood decay rates of each fungal strain were then compared across invertebrate and competing fungus treatments using two-way Analysis of Variance (Two-way ANOVA). All data were normally distributed (Anderson-Darling Test) and variances equal (Levene's Test) and individual treatments were compared using the Tukey pairwise comparison.

6.4 Results

6.4.1 Interaction outcomes in soil

All mycelial interactions were completed within 63 d. In the absence of grazers, extension rates of interacting fungi were negatively correlated with each other ($r = -0.601$, $n = 30$, $P <$

0.001); the growth of one competitor generally restricted development of its opponent. In ungrazed control trays there was a clear hierarchy of fungal dominance (*H. fasciculare* < *P. velutina* < *R. bicolor*). There was no difference between the competitive abilities of the two *H. fasciculare* strains with all replicates resulting in deadlock (Fig. 6.1). Both were consistently overgrown by *P. velutina*, and *R. bicolor* replaced all three competitors in the absence of grazing (Fig. 6.2).

Although invertebrates had no effect on the outcome of interactions between *H. fasciculare* and *P. velutina* (*P. velutina* overgrew both *H. fasciculare* strains in every treatment), all other interactions were significantly (Logistic Regression; $P \leq 0.05$) affected (Fig. 6.2). Isopods (*O. asellus*) preferentially grazed *R. bicolor* over *P. velutina* ($\chi^2_1 = 8.9$, $P = 0.003$) and *H. fasciculare* ($\chi^2_1 = 13.32$, $P < 0.001$). Selective grazing significantly ($P \leq 0.001$) reduced extension rates of *R. bicolor* compared to those in un-grazed controls (*R. bicolor* vs *H. fasciculare* JH: $F_{1,46} = 32.5$, $P < 0.001$; *H. fasciculare* DD3: $F_{1,46} = 103.59$, $P < 0.001$; *P. velutina*: $F_{1,46} = 30.39$, $P < 0.001$; Fig. 6.3), leading to its replacement in all *O. asellus* microcosms (Fig. 6.4). No other invertebrate species altered the outcome of interactions between *R. bicolor* and *P. velutina* (Fig. 6.2) but competition with *H. fasciculare* DD3 and *H. fasciculare* JH was significantly ($P \leq 0.05$) affected by *P. redivivus* and *F. candida*, respectively. The nematodes (*P. redivivus*) had no significant ($F_{1,46} = 3.25$, $P = 0.078$) effect on extension rates of *R. bicolor* but stimulated growth ($F_{1,46} = 5.05$, $P = 0.029$) and branching of *H. fasciculare* DD3 (Fig. 6.3, 6.4). This enabled the latter to overgrow its opponent in 60 % of *P. redivivus* microcosms and reversed the interaction outcome ($G = 10.044$, $P = 0.007$; Fig. 6.2). Despite showing a clear preference for *R. bicolor* over *H. fasciculare* ($\chi^2_1 = 21.33$, $P < 0.001$), collembola (*F. candida*) grazed extensively at the interaction zone between the two. This prevented either *R. bicolor* or *H. fasciculare* JH from reaching the opposing woodblock in 80% of microcosms (Fig. 6.2, 6.4). As neither fungus was removed, *F. candida* effectively ensured the survival of both species whereas *H. fasciculare* was replaced in all un-grazed microcosms. Millipedes (*B. guttulatus*) preferentially grazed *H. fasciculare* over *R. bicolor* ($\chi^2_1 = 5.4$, $P = 0.02$) and showed no preferences between the latter and *P. velutina* ($\chi^2_1 = 0.476$, $P = 0.49$). As *H. fasciculare* was the least competitive fungal species, *B. guttulatus* grazing did not significantly ($P > 0.05$) affect the outcomes of any fungal interactions (Fig. 6.2).

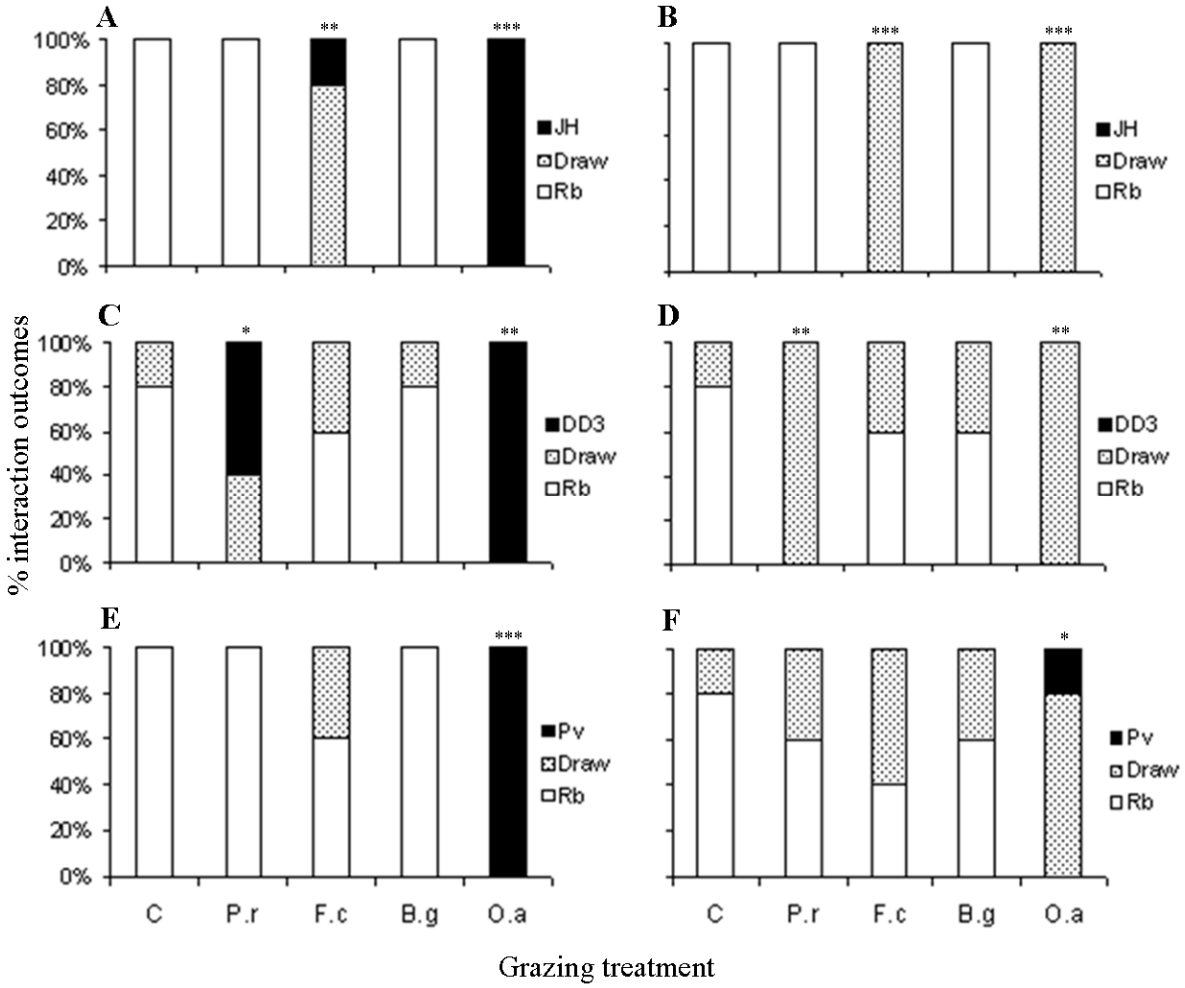

Fig 6.2: Percentage outcomes of competitive fungal interactions with *Resinicium bicolor* (Rb) against *Hypholoma fasciculare* JH (Hf JH), *Hypholoma fasciculare* DD3 (Hf DD3) and *Phanerochaete velutina* (Pv) in soil (A, C, E), and wood blocks (B, D, F) during control (C), *Folsomia candida* (F.c), *Oniscus asellus* (O.a), *Blaniulus guttulatus* (B.g) and *Panagrellus redivivus* (P.r) grazing treatments. Stars indicate significant differences (Logistic Regression) compared to un-grazed controls (***: $P \leq 0.001$, **: $P \leq 0.01$, *: $P \leq 0.05$). All other fungal interactions are not included as *P. velutina* out-competed both *H. fasciculare* strains in 100% of each grazing treatment and every interaction between the two *H. fasciculare* strains resulted in a draw.

6.4.2 Colonisation of wood blocks

The hierarchy of fungal dominance observed in soil was maintained in wood block colonisation. In the absence of grazing, *P. velutina* replaced *H. fasciculare* DD3 and JH, while all three were replaced by *R. bicolor* (Fig. 6.2). Invertebrates did not affect the colonisation of *H. fasciculare* (DD3 and JH) wood blocks by *P. velutina*; the former were replaced in every replicate. Grazing significantly ($P \leq 0.05$) affected wood block colonisation

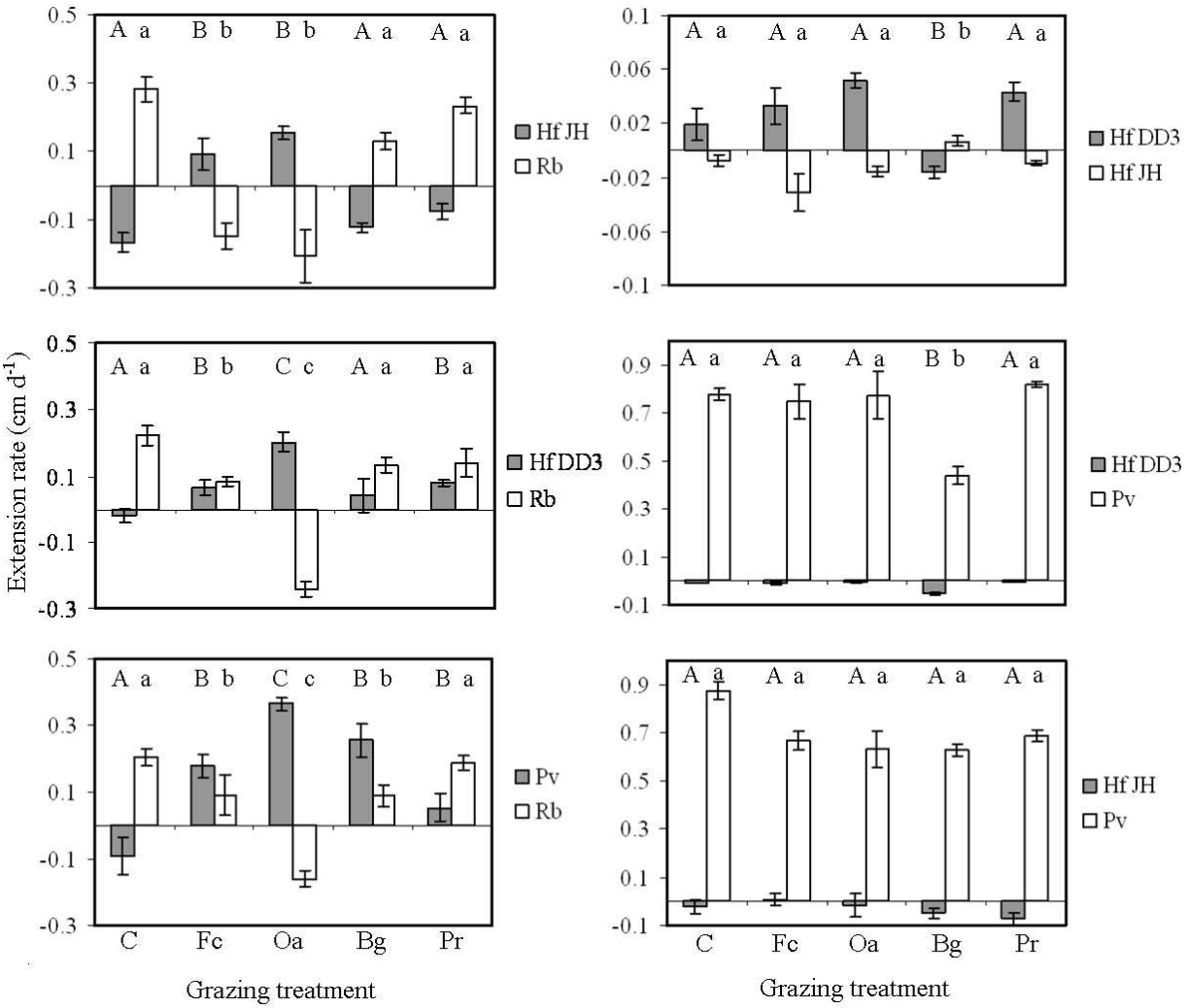

Fig. 6.3: Extension rates of *Resinicium bicolor* (Rb), *Phanerochaete velutina* (Pv), *Hypholoma fasciculare* DD3 (Hf DD3) and *Hypholoma fasciculare* JH (Hf JH) growing towards one another in control (C), *Folsomia candida* (Fc), *Oniscus asellus* (Oa), *Blaniulus guttulatus* (Bg) and *Panagrellus redivivus* (Pr) grazing treatments. Negative values indicate that mycelia are retreating due to grazing or competitive interactions. Different letters indicate significantly ($P \leq 0.05$; ANCOVA) different extension rates. Upper and lower case letters refer to different fungi and were analysed separately. y-axis scales vary between graphs.

during *R. bicolor* interactions but, unlike in soil, the interaction outcomes were not reversed (Fig. 6.2). *Oniscus asellus*, for example, grazed *R. bicolor* mycelia from the soil but could not access hyphae within wood blocks. Opposing fungi were, therefore, able to dominate the soil and encounter *R. bicolor* wood blocks, but were unable to displace the dominant competitor from its original resource. As a result, *O. asellus* grazing caused a shift from *R. bicolor*-dominated microcosms (both wood blocks colonised by *R. bicolor*) to those in which both

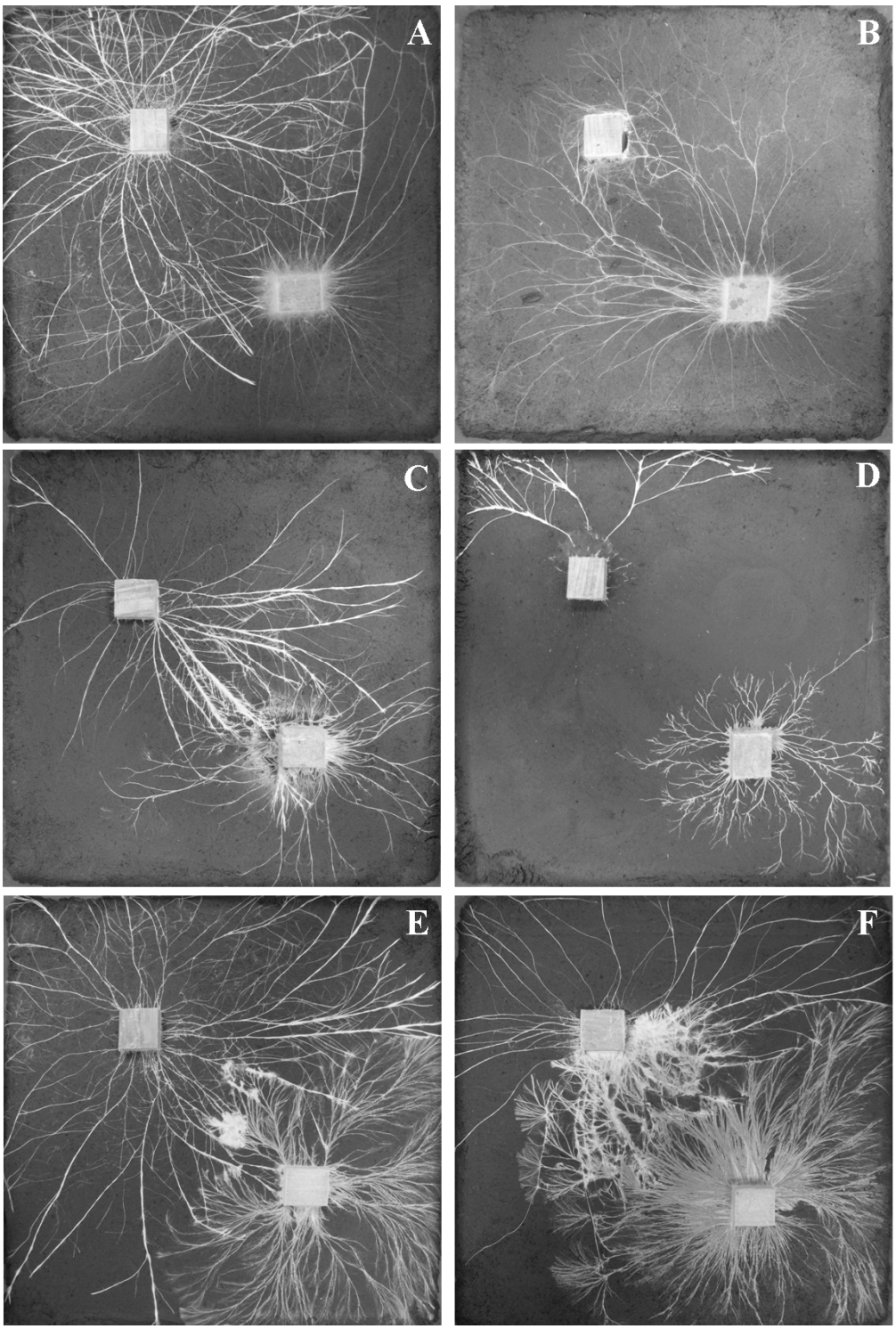

Fig. 6.4: Digital images showing the outcomes of mycelial interactions with *Resinicium bicolor* against *Phanerochaete velutina* (A, B), *Hypholoma fasciculare* JH (C, D) and *Hypholoma fasciculare* DD3 (E, F) in un-grazed control (A, C, E) and grazed (*Oniscus asellus* (B), *Folsomia candida* (D) *Panagrellus redivivus* (F) treatments. Competing mycelia extended from 2 x 2 x 1 cm wood blocks across 24 x 24 cm soil trays.

fungi survived (Fig. 6.2). *Panagrellus redivivus* had the same effect during interactions between *R. bicolor* and *H. fasciculare* DD3. By preventing either fungus from encountering opposing wood blocks, *F. candida* also shifted the balance between *R. bicolor* and *H. fasciculare* JH, allowing both fungi to co-exist in wood resources (Fig. 6.2).

6.4.3 Decay rates

In the absence of grazing, the decay rates of *P. velutina*-colonised wood blocks were influenced by the competing fungal species (Fig. 6.5). Decay rates were significantly ($F_{2,12}$ = 10.22, P = 0.003) faster during interactions with *Hypholoma fasciculare* JH and DD3 than when competing with *R. bicolor*. Decay rates of wood blocks colonised by the three remaining fungi were not significantly (P > 0.05) influenced by any opposing fungal strain.

The presence of grazers affected the rate of decay of wood blocks colonised by *P. velutina, R. bicolor* and *H. fasciculare* DD3 (Fig. 6.5). Significant (Two-way ANOVA; fungus*invertebrate interaction: P ≤ 0.05) interactive effects of competing fungus and grazing treatments suggested that fungal opponents determined the potential of grazers to affect wood decay rates. Overall decay rates were, however, positively correlated (r = 0.521, n = 300, P < 0.001) with mycelial extension rates of the colonising fungi. This suggests that any reduction in wood decay was the result of reduced mycelial growth, following intensive grazing or replacement by an opponent. During interactions between *R. bicolor* and *P. velutina,* decay rates of wood blocks colonised by the former were significantly ($F_{6,68}$ = 10.95, P = 0.014) reduced by *O. asellus* (Fig. 6.5). This also led to significantly ($F_{6,68}$ = 17.94, P = 0.003) increased decay rates of *P. velutina* wood blocks during the same interaction (Fig. 6.5). Decay rates of *R. bicolor* wood blocks were also decreased by *O. asellus* during interactions with *H. fasciculare* JH, but, unlike *P. velutina*, *H. fasciculare* JH decay rates were not significantly ($F_{6,68}$ = 24.21, P = 0.001) increased (Fig. 6.5). Whilst competing against *R. bicolor,* decay rates of *H. fasciculare* DD3 wood blocks were significantly (P ≤ 0.05) increased by *O. asellus* ($F_{6,68}$ = 10.19, P = 0.013) and *P. redivivus* ($F_{6,68}$ = 11.82, P = 0.009) grazing; the same two species which stimulated *H. fasciculare* DD3 extension across soil (Fig. 6.2). In contrast, *B. guttulatus* reduced *H. fasciculare* DD3 wood decay rates during intraspecific interactions with *H. fasciculare* JH ($F_{6,68}$ = 11.93, P = 0.01; Fig. 6.5). This is the only confirmed direct influence of grazers on decay rate; as neither opponent reached the opposing wood block, the decreased decay of *H. fasciculare* DD3 was the direct result of reduced fungal biomass during *B. guttulatus* grazing.

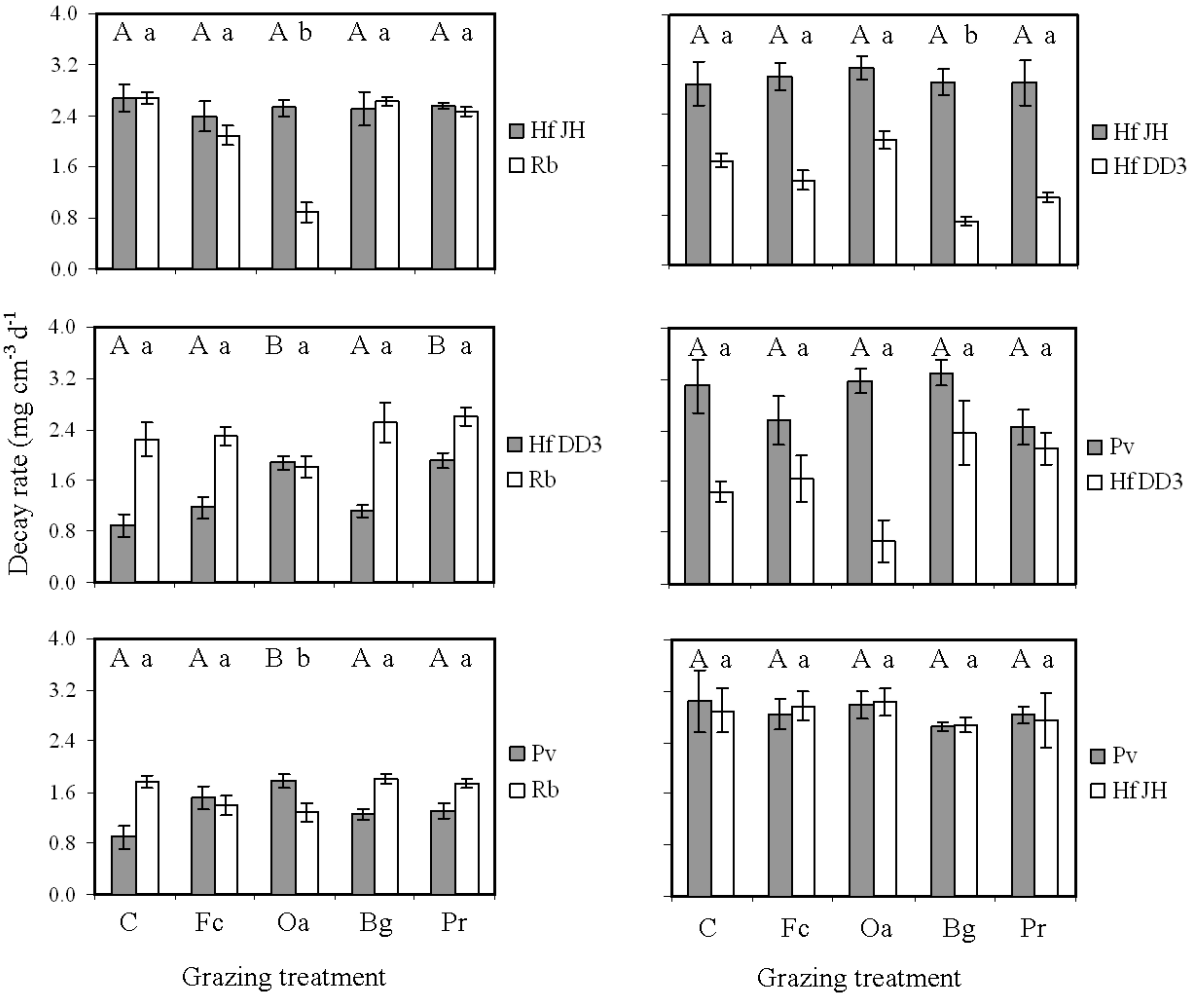

Fig. 6.5: Decay rates of wood blocks colonised originally by *Resinicium bicolor* (Rb) against *Hypholoma fasciculare* JH (Hf JH), *Hypholoma fasciculare* DD3 (Hf DD3) and *Phanerochaete velutina* (Pv) during competitive mycelial interactions against one another in control (C), *Folsomia candida* (Fc), *Oniscus asellus* (Oa), *Blaniulus guttulatus* (Bg) and *Panagrellus redivivus* (Pr) grazing treatments. Different letters indicate significantly ($P \leq 0.05$; Two-way ANOVA) different decay rates. Upper and lower case letters refer to different fungi and were analysed separately.

6.5 Discussion and conclusions

By differentially affecting the competitive abilities of interacting fungi, grazing invertebrates exerted selective pressures on fungal communities (supporting Hypothesis (i)). The potential for selective grazing to alter fungal community composition has been recognised since the 1970s (Parkinson *et al.* 1977) but has never been shown empirically. Although grazers are known to influence the relative abundances of litter fungi (Newell 1984a, 1984b), no previous

study has shown that grazing can reverse the outcomes of competitive mycelial interactions in soil. Grazers can determine fungal dominance via two opposing mechanisms: (i) restriction of the more competitive fungus; or (ii) stimulation of the less competitive species. Here, extensive, selective grazing of the dominant fungus (*R. bicolor*) by *O. asellus* populations prevented the competitive exclusion of opponents, while the stimulated, 'compensatory' growth of *H. fasciculare* during *P. redivivus* feeding (recorded previously in this species and probably the result of mobilisation of storage compounds and increased nutrient uptake from wood resources to counteract the negative effects of grazing; Chapter 3) enabled it to overcome a more competitive opponent by a process of gross mycelial contact (Boddy 2000). Their ability to alter fungal dominance suggests that, along with litter type and soil quality (Bardgett 2005), grazing invertebrates represent a key factor determining the community composition of saprotrophic fungi in soil and wood resources. This will have direct implications for nutrient mineralisation and cycling. *Phanerochaete velutina*, for example, produces more cellulytic enzymes (Chapter 5), and decomposes wood at a faster rate (Tordoff *et al.* 2008) than *R. bicolor* when growing alone. The potential of *O. asellus* to shift fungal communities in favour of the former may, therefore, lead to increased nutrient mineralisation and wood decomposition rates.

Top-down determination of community composition has been well documented in aboveground terrestrial and aquatic ecosystems, as well as belowground bacterial-based communities (Wardle & Yeates 1993; Veen *et al.* 2010). These effects are commonly associated with high intensity grazing exerting strong selective pressures. Prior to this study, the lack of evidence supporting this process in fungal communities may have been due to absence of macrofauna in empirical studies; previous work on the effects of grazers on fungal community structure have been limited almost exclusively to micro- and mesofauna (Nematoda, Collembola and Oribatida). In the present study, *F. candida* and *P. redivivus* affected the development of specific fungal interactions but *O. asellus* exerted the strongest selective pressures on competing fungi. This supports Hypothesis (ii) and highlights the Isopoda as a particularly important group of decomposers, not only through the communition (shredding and digestion) of litter, but also through their modification of fungal activity (Bradford *et al.* 2002; Hättenschwiler *et al.* 2005). Along with *Porcellio scaber, O. asellus* is one of the most abundant terrestrial isopods in temperate ecosystem soils (Jones & Hopkin 1996). Even at low density, both species are capable of selectively removing entire mycelial cord systems from soil microcosms (Chapter 3), highlighting their potential capacity to

regulate fungal abundance, community composition and diversity in natural and agricultural systems (Mitschunas *et al.* 2006).

Even within invertebrate size groups, grazers varied in their effects on interacting fungi. Unlike isopods, millipede (*B. guttulatus*) populations selectively grazed the least competitive fungi (*H. fasciculare* JH and DD3) and, as a result, had no effect on the outcomes of fungal interactions. Contrasting feeding preferences among mycophagous soil fauna have rarely been recorded; most studies report similar preferences for palatable or nutritious fungal resources (e.g. dark pigmented fungi; Maraun *et al.* 2003 and references cited therein). *Hypholoma fasciculare* and *P. velutina* are generally considered unpalatable resources due to their production of sesquiterpenes - secondary metabolites, commonly used in defence against fungivores (Hynes *et al*. 2007). These fungi were avoided by *O. asellus* and *F. candida,* but the apparent tolerance of *B. guttulatus* may represent an example of resource partitioning (Maraun *et al.* 2003; Setälä *et al.* 2005), enabling the coexistence of various decomposer invertebrate species within similar soil environments. Species-specific feeding behaviour suggests that predicted changes to soil invertebrate diversity and taxonomic make-up brought about by global climate change (Jones *et al.* 1998; Wolters *et al.* 2000) will have direct consequences for fungal community composition. While abiotic climate change parameters (e.g. elevated temperature and rainfall) directly affect microbial activity (Gange *et al.* 2007) and community composition (Fierer *et al.* 2003; He *et al.* 2010), the indirect effects, mediated through changes in the biological community, may be equally important for the functioning of the decomposer subsystem (Gessner *et al.* 2010).

The aforementioned changes in fungal community structure are likely to influence litter decomposition rates (i.e. if a slow decomposer fungus is replaced by a more rapid species, overall decay rates will be increased). The changes in wood decay rates highlight that the impacts of grazing may not be quite as straightforward. For example, decay rates by *R. bicolor* were reduced during extra-resource mycelial grazing by *O. asellus*. This effect has been recorded previously (Tordoff *et al.* 2008; Chapter 4), but in the present study, the removal of *R. bicolor* also stimulated mycelial growth and decomposition rates by *P. velutina*. Resulting decay rates were not only increased via the change in fungal community composition, but also by the stimulated growth and activity of the promoted fungal species during grazing. This suggests that, by modifying the development of one mycelial system, high intensity grazing events may alter the activities of all interacting species within the local

fungal community. In conclusion, selective grazing by soil fauna will affect rates of nutrient turnover, both by altering fungal species composition, and also by modifying the relative capacities of interacting fungi to decompose wood.

7. Impacts of grazing soil fauna on decomposer fungi are species-specific and density-dependent

7.1 Abstract

The grazing impacts of different densities of woodlice, collembola and millipedes on the foraging and distribution of two saprotrophic cord-forming basidiomycetes were investigated in soil microcosms. Effects of all three invertebrate species were density-dependent, with larger populations limiting mycelial development to a greater extent. Impacts were, however, species-specific; grazing pressures exerted by low-density woodlouse populations outweighed those of high-density millipede or collembola populations. The varying abilities of soil invertebrates to influence mycelial foraging and distribution indicate that invertebrate species composition and diversity may be key factors regulating saprotrophic basidiomycete functioning in woodland soil.

7.2 Introduction

Interactions between soil fungi and invertebrates have received considerable attention as both groups represent major trophic and functional components of the soil environment (Tordoff *et al.* 2006; Wood *et al.* 2006). Primary decomposition in forest ecosystems is dominated by saprotrophic cord-forming basidiomycetes (Boddy & Watkinson 1995). These fungi are non-unit-restricted; dynamic mycelial networks link resources over large distances (Boddy 1993) and reallocate nutrients across heterogeneous soil environments (Boddy 1999). As a consequence of their large biomass and relatively low C:N ratios (Swift & Boddy 1984), these networks represent the primary nutrient source for soil invertebrates (Pollierer *et al.* 2009). Grazing can influence the foraging and activity of basidiomycete systems.

Impacts of soil invertebrate grazing on cord-forming fungi are taxon-specific; certain macrofauna (e.g. woodlice) can remove entire mycelial systems, whereas mesofauna (e.g. collembola, enchytraeids) and microfauna (e.g. nematodes) have less extreme effects (Chapters 3 and 4). Within taxa, species-specific impacts are also evident, such as those elicited by different collembola (Kampichler *et al*. 2004; Tordoff *et al*. 2008). Species-specific grazing preferences (Chapter 6) give mycophagous fauna the potential to drive, and respond to, changes in fungal community composition (Klironomos *et al*. 1992; Jones *et al*. 1998). Intense grazing on the dominant combatant can, for example, prevent the competitive exclusion of less-competitive species, potentially driving changes in fungal species composition and diversity (Chapter 6).

The impacts of grazers are also density-dependent. At low population densities, mycophagous collembola stimulate mycelial growth, but inhibitory affects increase once grazer density reaches a certain threshold (Bengtsson & Rundgren 1983; Hanlon & Anderson 1979; Bretherton *et al*. 2006). It is unclear whether this will mask the species-specific effects; high-intensity micro- or mesofauna grazing may outweigh the effects of smaller macrofauna populations. Climate change is predicted to drive shifts in the abundance and species composition of soil invertebrates (Jones *et al*. 1998; Briones *et al*. 2009). Identifying how these changes are likely to influence fungal distribution and function may be key to our understanding of fungal-mediated nutrient mineralisation and decomposition.

The aim of this study was to determine whether the impacts of different densities of mycophagous invertebrate taxa mask the detection of species-specific efects on mycelial development of cord-forming basidiomycetes. Three specific hypotheses were tested: (i) grazing by soil invertebrates will elicit observable density-dependent responses in the experimental fungi; (ii) species-specific impacts will be evident at comparable representations of grazer field density; and (iii) density-dependence will mask the detection of species-specific impacts overall.

7.3 Materials and methods

7.3.1 Invertebrate collection and culturing

Folsomia candida (Collembola; Cardiff University Culture Collection) were cultured on a medium of 95% plaster of Paris (Minerva Dental, Cardiff, UK) and 5% activated charcoal (Sigma, Poole, UK) and fed dried baker's yeast (Spice of Life, Cardiff, UK). *Oniscus asellus* (Isopoda) and *Blaniulus guttulatus* (Diplopoda) were collected from Coopers Field, Cardiff. Invertebrates were size-selected (Table 7.1) and starved for 24 hr prior to experimental use. Three densities (low, medium and high; Table 7.1) were employed, representing low- to mid-range estimates of field densities (Peterson & Luxton 1982; Topp *et al.* 2006) as grazers were restricted to a 2-D habitat.

Table 7.1. Selected size range, field density range and experimental densities of the invertebrate grazer species.

	Size range	Field density range (m^{-2})	Experimental density (m^{-2}) (number per microcosm)		
			Low	Medium	High
Oniscus asellus	≥ 0.5 cm	35-630 (Topp *et al.* 2006)	33.2 (2)	83 (5)	166 (10)
Blaniulus guttulatus	≥ 0.5 cm	15-230 (Petersen & Luxton 1982)	33.2 (2)	83 (5)	166 (10)
Folsomia candida	200-400 µm	100-67 x 10^4 (Petersen & Luxton 1982)	313.2 (25)	783 (60)	1566 (120)

7.3.2 Fungal culturing and inoculum preparation

The cord-forming basidiomycetes *Resinicium bicolor* and *Phanerochaete velutina* were cultured on 2% malt agar (MA). Wood blocks (2 × 2 × 1 cm), obtained from freshly-felled beech (*Fagus sylvatica*) and frozen until required, were defrosted in de-ionised water (DH_2O)

for 12 hr. They were then heat-sealed in two layers of autoclave plastic and autoclaved (121°C) three times at 24 hr intervals. Sterile blocks were placed on 14 cm diameter Petri dishes containing MA colonised with the experimental fungi and incubated in darkness at 20 ± 1°C for 3 months.

7.3.3 Microcosm preparation and inoculation

Topsoil from mixed deciduous woodland (Coed Beddick Enclosure, Tintern, UK (see Chapter 3 for location details)) was sieved using a 10 mm mesh, air-dried in the laboratory for 5 d, sieved using a 2 mm mesh and frozen for 24 h. Frozen soil was mixed with DH_2O (340 ml kg^{-1}). Lidded clear plastic trays (Nunc-Gibco, Paisley, UK; 24 × 24 cm, 2 cm deep) containing 200 g of soil, compressed to a 5 mm depth, were centrally inoculated with colonised wood blocks and incubated at 20 ± 1°C. These were weighed on inoculation and sprayed with DH_2O every 7 d to replace moisture loss. Invertebrates were added when the mycelial diameter had reached 8 cm in 50% of replicates (6 and 11 d after inoculation for *P. velutina* and *R. bicolor*, respectively). All treatments were replicated five times.

7.3.4 Image capture and analysis

Images were captured using a Nikon Coolpix 7500 digital camera prior to invertebrate addition and when control mycelia were 2 cm from microcosm edges (8 and 12 d for *P. velutina* and *R. bicolor*, respectively). Images were captured at a height of 39.5 cm under laboratory lighting and analysed using ImageJ. Radial extent and hyphal coverage were measured (see Chapters 3, 4 and 5).

7.3.5 Statistical analysis

Analyses were completed in R (R Development Core Team 2008). Change in radial extent and hyphal coverage were analysed using a 2-way Analysis of Variance (GLM), with invertebrate species and density as factors. For each invertebrate species, density impacts were analysed using one-way ANOVA and Tukey's pairwise comparisons. Radial extent data for *P. velutina* were transformed by √ (change in extent + 2) to achieve normality.

7.4 Results, discussion and conclusions

For both fungal species hyphal coverage data mirrored radial extension (Fig. 7.1). By slowing extension, all three grazers reduced the area of fungus-colonised soil. Impacts were

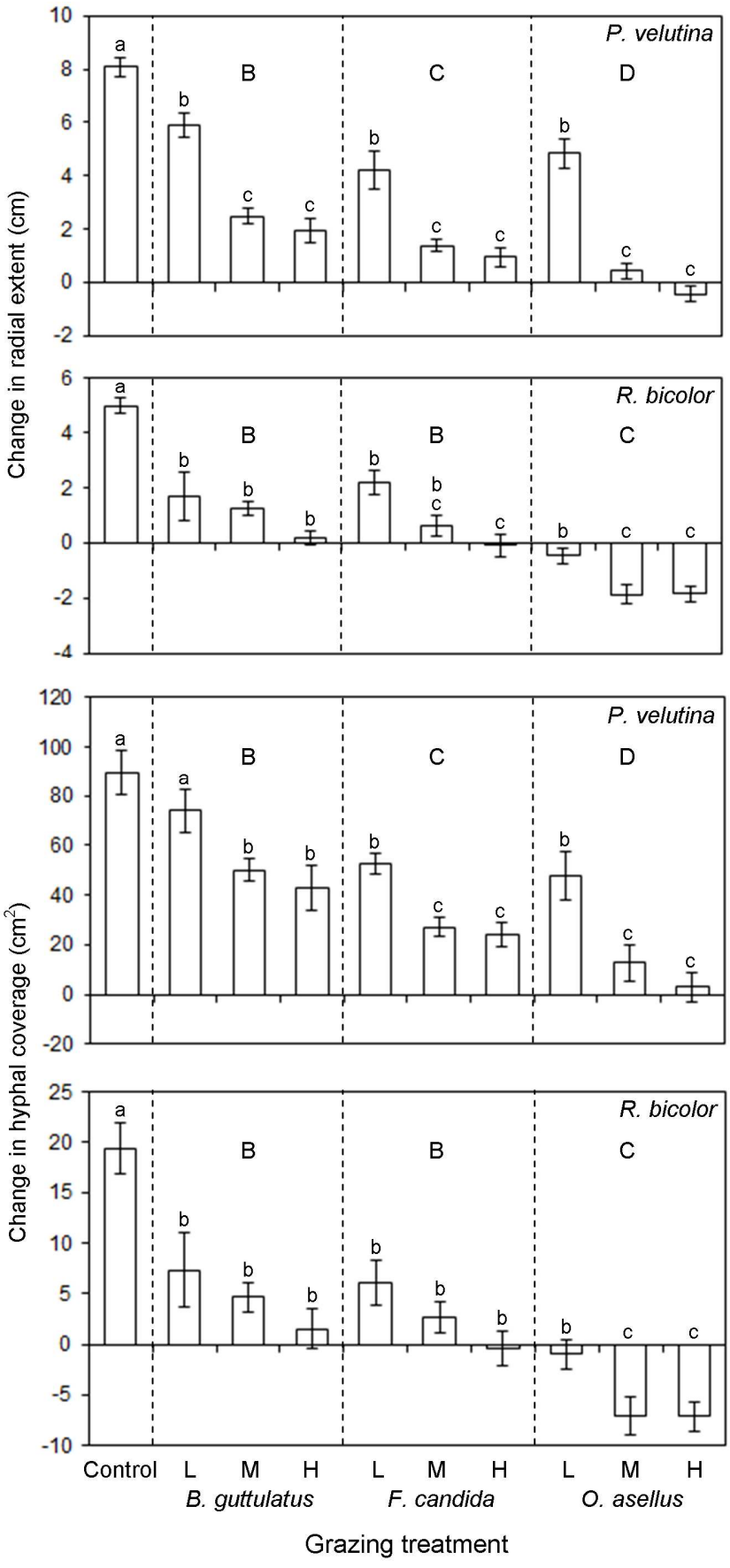

Figure 7.1. Impacts of grazing by *Blaniulus guttulatus*, *Folsomia candida* and *Oniscus asellus* at low (L), medium (M) and high (H) density on changes in radial extent and hyphal coverage of *Phanerochaete velutina* and *Resinicium bicolor*. Uppercase letters indicate significant ($P \leq 0.05$) species-specific grazer impacts (GLM). Lowercase letters indicate significant ($P \leq 0.05$) density-dependent impacts of the grazer concerned.

density-dependent (GLM; *P. velutina* extension: $F_{2, 36} = 79.659$, $P < 0.001$, coverage: $F_{2, 36} = 22.879$, $P < 0.001$; *R. bicolor* extension: $F_{2, 36} = 12.503$, $P < 0.001$, coverage: $F_{2, 36} = 6.981$, $P = 0.003$), supporting Hypothesis (i) and previous grazing studies (Bengtsson & Rundgren 1983; Hanlon & Anderson 1979; Bretherton *et al.* 2006). Low-density populations consistently exerted the weakest grazing pressures but medium- and high-intensity grazing effects were not significantly (ANOVA; $P \geq 0.05$) different. Effects of the largest invertebrate populations were limited by resource availability (five *O. asellus* removed entire *R. bicolor* systems so 10 individuals could not have had a greater effect) suggesting that grazing impacts level off as density exceeds a certain threshold. This trend did not vary between the three invertebrate species (Invertebrate*Density interaction; $P \geq 0.05$) for both fungal growth parameters and is, therefore, likely to reflect the impacts of a wide variety of soil fauna. The apparent relationship between mycelial growth and enzyme production during grazing (Chapter 5) suggests that factors which contribute to high soil invertebrate abundance (e.g. increased temperature and precipitation) will not only restrict the ability of fungi to forage for nutrients, but also to decompose organic resources after acquisition. The varying, or patchy, distribution of most soil invertebrates suggests that mycelial functioning may also vary at local scales. At microsites of faunal aggregation, intense grazing may be particularly important in regulating fungal-mediated nutrient distribution and decomposition in soil (Kaneko *et al.* 1998).

Despite this density-dependent trend, the over-riding effects were strongly species-specific (GLM; *P. velutina* extension: $F_{2, 36} = 18.414$, $P < 0.001$, coverage: $F_{2, 36} = 21.63$, $P < 0.001$; *R. bicolor* extension: $F_{2, 36} = 30.892$, $P < 0.001$, coverage: $F_{2, 36} = 18.702$, $P < 0.001$), supporting acceptance of Hypothesis (ii) and rejection of Hypothesis (iii). Hyphal coverage of *R. bicolor* systems were, for example, lower during medium- and low-intensity *O. asellus* grazing than during high-intensity *F. candida* grazing (Fig. 7.1). This may relate to varying body and/or mandible size, although the differential impact of isopods and millipedes (macrofauna species of similar size) suggests that other factors, such as invertebrate feeding preferences and metabolic rate, may also influence grazing potential (Chapter 3). This highlights that, along with resource availability (Boddy 1999; Moore *et al.* 2003), invertebrate species composition may be a key factor influencing the foraging and distribution of basidiomycete systems in soil. Predicted changes to collembola, woodlouse and millipede species compositions in response to global climate change (Jones *et al.* 1998; Wolters *et al.* 2000) will, therefore, have direct consequences for basidiomycete growth and activity in soil. Investigating the grazing

potential of a wider variety of taxa may improve substantially our understanding of key processes influencing organic matter decomposition and nutrient cycling.

8. General discussion

8.1 Synthesis

The studies reported in this dissertation contribute to our understanding of two major components of the soil ecosystem (saprotrophic fungi and soil invertebrates), the interactions between them and the consequences for wood decomposition. To date, the majority of work concerning fungus-grazer interactions has neglected the diversity of belowground invertebrate communities. Although mites and collembola (mesofauna) represent an important component of mycophagous invertebrates, the present empirical studies are among the first to highlight the key roles of micro- and macrofauna in determining fungal growth and functioning. They also reflect the variable and, somewhat idiosyncratic nature of the soil environment; effects vary between and within invertebrate and fungal species. On its own, this finding highlights the importance of including a variety of taxa, representing different size or functional groups, to identify general trends in belowground ecology.

The intense grazing of mycelial cords by woodlice and millipedes was the key finding of Chapters 3 and 4. Macro-detritivore preferences for litter material colonised or "conditioned" by fungi has been recorded previously (Maraun *et al.,* 2003), but the current studies provide the first evidence of their potential (woodlice in particular) to limit, and even prevent mycelial growth. Mycophagy in these species is not surprising considering the tendency of both invertebrate groups to aggregate in or underneath the woody litter substrata from which basidiomycete fungi obtain their nutrients and extend (Topp *et al.,* 2006). The effects observed highlight macrofauna as a particularly important group influencing mycelial abundance and fungal-mediated distribution of nutrients in forest soil. Grazing by macrofauna during mycelial emergence is likely to be a rate-limiting step in the development of basidiomycete fungi in woodland soil. High intensity grazing is not only likely to limit the ability of basidiomycete fungi to decompose wood, but also to extend in search of fresh resources.

Chapter 5 reinforced this finding by identifying the potential of these grazer species to influence fungal enzyme production. Considering the key role of extracellular enzymes, not only in the acquisition of nutrients by fungi, but in the decomposition process, it is surprising

that the effects of grazers have been largely overlooked. While previously accepted that the presence of invertebrates can influence enzyme activity in soil and litter (Mikola *et al*., 2002; Hättenschwiler *et al.,* 2005), the specific nature of enzymatic responses (occurring mostly in those associated with C and P cycling, for example) were unknown. Moreover, the opposing responses of fungi displaying explorative and exploititive foraging strategies highlighted further the importance of fungal community composition in determining the mineralisation and cycling of forest floor nutrients.

The variation in fungal susceptibility to grazing (recorded in Chapters 3, 4 and 5) encouraged the re-visiting of an 'old' question in soil ecology. Since the 1970s it has been predicted that differential effects of grazers on competing fungi can alter community composition (Parkinson *et al.,* 1979), but no empirical studies had categorically shown this. It was thought that the large size and unpalatable nature of fungi (especially cord-formers) limited the potential of grazers to control the outcomes of competitive interactions, but the intense grazing by macrofauna suggested that they might exert a selective pressure. In Chapter 6 heavy grazing of *R. bicolor* by woodlice reversed the outcomes of competitive mycelial interactions. Unexpectedly, nematodes also reversed interaction outcomes by an opposing mechanism - stimulating growth of a formerly less competitive fungus over its opponent. This top-down determination of fungal community composition may represent one, if not the most important role of soil invertebrates in woodland ecosystem functioning.

8.2 Microcosm studies

Most of the results presented were consistent with previous microcosm studies: (1) grazing usually reduced mycelial coverage, especially in palatable species such as *R. bicolor* (Tordoff *et al.* 2006); (2) low intensity grazing stimulated mycelial growth, especially in fast-growing species such as *H. fasciculare* and *P. velutina* (Bretherton *et al.* 2006); and (3) grazing influenced fungus-mediated wood decay rates (Harold *et al.* 2005). These suggest that results from microcosms as used in these studies are reliable and repeatable. The simplicity of microcosms does not, however, account for several layers of complexity, which occur in natural soil ecosystems and may influence the interactions observed *in vitro*.

8.2.1 Three-dimensional soil environment

The 2-D setup allowed the observation of interactions which would have been obscured by the 3-D nature of natural soil habitats. While bio-indicators such as ergosterol or phospholipid fatty acids (PLFAs) may give some measure of microbial biomass (Mille-Lindblom *et al.* 2004), identifying changes to fungal morphology and network architecture is precluded in 3-D soil or litter studies. The simplistic 2-D microcosms used in the present studies do, therefore, provided a unique means of identifying invertebrate grazing potential and the specific growth responses of fungal mycelia. Results must, however, be approached cautiously as the stratified nature of 3-D soil may limit contact between species forced to interact in 2-D. Leonard and Anderson (1991) showed that 3-D soil microcosms provided refugia for fungal hyphae and limited the grazing effects of collembola compared to those in 2-D soil. This highlights the need to move towards 3-D microcosms to uncover the true potential of soil invertebrates in influencing fungal growth and functioning.

8.2.2 Complex invertebrate food webs

To identify and compare the effects of individual species, invertebrates were always used in solitude. Interactions (competitive, predatory etc.) between the considerable diversity of soil invertebrate species were ignored. These interactions are likely to influence grazing effects; predation and competition for resources are likely to reduce the impacts of individual species. The activity of predatory mites, for example, has been shown to restrict fungal grazing by collembola, *Folsomia fimetaria* (Hedlund & Ohrn 2000). Similarly, the impacts of small invertebrates can be negated by the effects of larger, more destructive species competing for similar resources in complex environments (Bradford *et al.* 2002). Increasing the number of decomposer species or trophic levels may provide valuable insights into the role of invertebrate community structure on ecosystem functioning. Removing individual species from multi-species environments may provide more accurate estimations of the effects of those species on mycelial growth and functioning.

8.2.3 Limited alternative resources

In the simplistic 2-D microcosm environments used in these studies, grazers lacked the range of alternative resources that would be available to them in the field. Preferences of various invertebrate species for dark pigmented fungi over saprotrophic basidiomycetes (Maraun *et al.* 2003), for example, may suggest that they selectively avoid the latter when given the opportunity in the field. Several studies have also revealed preferential grazing by soil fauna

on microfungi over arbuscular mycorrhizal and saprotrophic fungi (see Gange 2000). Unlike ephemeral microfungi, the thick cords of saprotrophic basidiomycetes are relatively long-lived and vital to nutrient translocation and exploratory growth. The severing of cords by invertebrates is likely to be extremely costly to these fungi. It is, therefore, possible that cords have evolved to be unpalatable or resistant to gazers. These resources may be ignored in natural ecosystems, with a vast abundance of alternative microbial and litter resources available. The study by Newell (1984a), however, provides some evidence that collembola do graze, and affect distributions of basidiomycetes in the field.

Limited resource availability is also likely to have had consequences for invertebrate fitness. Survival and reproduction of soil invertebrates, particularly polyphagous species, are often dependent on the availability of a range of nutrient sources. Even whilst grazing their preferred fungal resource (*Cladosporium cladosporioides*), *F. candida* fitness was reduced in single, compared to mixed diet environments (Janssens *et al.* 2010). The importance of mixed diets has also been shown in other invertebrate guilds, with reduced fitness of carabid beetle (Symondson *et al.* 2006) and linyphiid spider (Harwood *et al.* 2009) predators when limited to feeding on single prey species. Reduced grazer fitness, resulting from limited resource availability, is likely to have reduced grazing effects recorded in microcosms.

8.2.4 Limited fungal resources

Resources were also limited from a fungal perspective. In the field, basidiomycete mycelia will encounter numerous heterogeneously distributed litter resources leading to the formation of large, persistent networks (Boddy 1993). Following acquisition of fresh resources, the formation of thick interconnecting cords is accompanied by the regression of diffuse, unconnected hyphae (Boddy 1999). These thick, highly lignified cords are less susceptible to grazing collembola in soil microcosms (Wood *et al.* 2006). Formation of cross-links between mycelia often accompanies cord formation, further increasing the resilience of networks to attack by mesofauna (Boddy *et al.* 2010). It currently remains unclear whether larger, more destructive grazers will affect these more established systems (macrofauna, for example, appear to prefer grazing large mycelial cords; Chapter 3) but the availability of alternative litter resources in the field is likely to increase the resilience of mycelial networks to grazing, beyond that recorded in simplistic microcosms.

8.3 Future directions

Section 8.2 highlights some of the limitations of using simplified microcosms to represent processes in the field. The potential to control for external abiotic and biotic factors, however, suggests that these *ex situ* studies provide valuable insights into specific interactions and mechanisms which are obscured by the complexity of the natural soil environment. The use of mesocosms - sections of untreated soil including all the natural complexity, maintained under laboratory conditions - may prove to be the next step in bridging the gap between microcosms and the field. Although concerns have also been raised regarding the reliability of results obtained from mesocosm experiments (Lawton 1996), effects recorded in 3-D, multi-species environments are likely to more closely resemble those in the field. The tendency of mycelia to grow below the soil surface (i.e. out of view) does, however, present the major barrier to the use of mesocosms for investigating factors affecting fungal development. The use of stable isotope tracers has recently been employed to determine the effects of grazers on N partitioning within mycelial systems growing in 2-D microcosms (Tordoff *et al.* 2011). This could be used in more complex systems to identify changes in belowground mycelial growth and fungal-mediated nutrient dynamics. Furthermore, the use of next-generation molecular profiling techniques (e.g. pyrosequencing, qPCR or GeoChip) can provide valuable insights into microbial biomass, community compositions and functional gene diversity (He *et al.* 2010). The use of mesocosms coupled with molecular and stable isotope techniques will undoubtedly be the next step in identifying the true effects of grazing soil fauna on microbial activity and functioning.

The taxon-specific effects of grazers suggest that conditions which contribute to changing invertebrate communities will alter mycelial functioning. By modifying invertebrate activity and species composition, climate change factors are likely to indirectly influence their fungal resources (Jones *et al.* 1998; Briones *et al.* 2009; Day *et al.* 2009). Investigating the direct effects of elevated CO_2, water potential, temperature and O_3, alone and in combination, on fungus-invertebrate interactions is likely to provide valuable insights into the effects of climate change on belowground ecosystem functioning and carbon storage. Elevated temperature, for example, is known to stimulate growth and decomposition rates of most basidiomycete fungi (Boddy 1983), but the regulatory effects of high intensity grazers (Chapter 4, 6) may mitigate these effects (Crowther *et al.* 2012). Investigating the interactive

effects of biotic and abiotic factors may be key in determining the effects of climate change on belowground ecosystem processes.

A final question raised in Chapter 6 relates to the potential of grazers to influence wood decay rates by un-grazed mycelial systems. By feeding on *R. bicolor*, woodlice indirectly increased the growth and wood decay rates of the competing fungus, *P. velutina*. This suggests that, by modifying the development of one mycelial system, high intensity grazing events may alter the activities of all interacting fungi within the local community. Exploring this concept further will give insights into the effects of grazers on the functioning of entire microbial communities. By restricting the movement of grazers within multi-species fungal microcosms, it may be possible to track the extent of grazing effects (using stable isotope tracers or measuring decomposition rates) and realise the full potential of invertebrates to affect woodland ecosystem functioning.

9. References

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